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COLIFORM BACTERIA

LELAND W. PARR

Department of Bacteriology, Hygiene and Preventive Medicine, School of Medicine, The George Washington University, Washington, D. C.

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The oldest members of the coliform group of bacteria, as the reviewer conceives it, are *Klebsiella pneumoniae*, or Friedländer's bacillus, described in 1882, and *K. rhinoscleromatis*, which v.Fritsch recorded the same year. Next come *Escherichia coli* and *Aerobacter aerogenes*, both of which were ushered into the bacteriological world in 1885 by Escherich. Somewhat younger is *A. cloacae*, described in 1890 by Jordan. *Proteus morgani*, the problem child of the group, dates from 1908 and the juveniles are *E. freundii*, recorded in Braak's Delft thesis of 1928, and *K. paralytica*, the etiological agent of "moose disease," described in 1932 by Cahn, Wallace, and Thomas.

These are the principal members of the coliform group as listed in the fifth edition of Bergey's Manual of Determinative Bacteriology (12), a great simplification of the genera *Escherichia*, *Aerobacter* and *Klebsiella* given in the fourth edition (1934)

which contained 35 species. In the latest edition these three genera comprise but ten species.[1]

With this simplification we are in heartiest accord, but the problem has several aspects. Among them, the principle of simplification, or "lumping," as Skinner and Brudnoy (149) term it, must be defended. The inclusion of the genus *Klebsiella*[2] with the coliform bacteria has to be explained. To claim the Morgan bacillus as a coliform organism will require justification. The broadening of the concept of *Escherichia coli* to include such forms as *E. coli-mutabile* and the paracolon bacilli which ferment lactose slowly or not at all is a new development and there are many who will want to know how the "coli-aerogenes intermediates" (180, 165, 25, 123) are classified and why.

THE TERM "COLIFORM"

The term "coliform" has long been in use by British bacteriologists (4, 23, 59, 92, 100, 132, 133, 160). In America, Breed and Norton (16) suggested the term to describe the lactose-fermenting bacteria used as a measure of the pollution of water. In 1937 H. E. Jordan advised that as Editor of the Journal of the American Water Works Association his policy would be to substitute "coliform" bacteria for "*B. coli*" or "colon group" in papers submitted to him (70). As Jordan also stated, the term "*coli-aerogenes*" continues as official in water analysis since the eighth edition of Standard Methods of Water Analysis (157) uses it. And "*Escherichia-Aerobacter*" is also official since that is the terminology employed in the sixth edition of Standard Methods of Milk Analysis (156).

SEPARATION OF FRIEDLÄNDER GROUP FROM OTHER COLIFORM BACTERIA

As noted above, the first members of the coliform group to be described were *Klebsiella pneumoniae*, from acute fibrinous

[1] The other three species are *Klebsiella ozaenae*, described by Abel in 1893; *K. granulomatis* of Aragão and Vianna (1912); and *K. capsulata* recorded in 1889 by Pfeiffer.

[2] These organisms have also often been called the "*Bacillus mucosus capsulatus* group."

pneumonia, and *K. rhinoscleromatis*, from rhinoscleroma. The typhoid bacillus had been described less than two years earlier and the emphasis in early bacteriology on its medical aspects favored the finding of pathogens. But even at this time the inclusion of the encapsulated forms found in the upper respiratory tract in a group with coliform bacteria from the intestine and from milk was urged (31). From the first all studies of the gram-negative, aerobic, encapsulated bacilli, of which the Friedländer bacillus is the type known to pathologists, have also included *Aerobacter aerogenes*[3] and all studies of coliform bacteria have likewise included *A. aerogenes*.

The close relationship of *Klebsiella* and *Aerobacter* has been asserted or demonstrated down to the present time (72, 74, 75, 76, 77, 41, 59, 170, 93). Perkins maintained that the prototype of the encapsulated group was *A. aerogenes* and that the other members were variants which had lost the power, in whole or in part through modification in environment (125), to ferment certain sugars. Edwards stated that *A. aerogenes* is so closely related to the other encapsulated forms that they should be classified in the same genus (41).

Notwithstanding, the Friedländer group and the rest of the coliform bacteria have been kept apart in the minds of most bacteriologists. This unfortunate point of view has separated two groups which must be considered together and it has minimized our understanding of the potentialities for pathogenicity possessed by *Escherichia* and *Aerobacter*. As we shall see, these forms, particularly *Escherichia*, are important in pathology, especially in infections of the urinary tract, in all age groups, and of the gastro-intestinal tract in the very young.

As a result we shall have to trace the steps in the history of the classification of coliform bacteria bearing in mind that *Klebsiella* is probably not under consideration and that the emphasis is mainly on the sanitary aspects of the organisms in question. Later we shall bring the Friedländer group back into the dis-

[3] In our own discussion in this review the terminology of the Bergey Manual will be employed but we believe that the use of one genus for all coliform bacteria is biologically more sound than the use of three.

cussion for it has a definite place in the concept of the coliform group.

DEVELOPMENT OF THE CLASSIFICATION AND CHARACTERIZATION OF COLIFORM BACTERIA

Escherich (42, 43) characterized *Bacterium coli-commune* as a bacillus of feeble motility which coagulated milk but did not liquefy gelatin; which fermented milk-sugar and grape-sugar with the disengagement of gas; which produced a moist growth on potato of a color varying from corn yellow to pea yellow; and which produced in animals a rapidly fatal disease characterized by diarrhea, somnolence and coma. *Aerobacter aerogenes* was first described as *Bacterium lactis-aerogenes* by Escherich who noted that it was shorter and plumper than *B. coli*, coagulated milk more actively and was non-motile. He stated that it fermented milk-sugar, cane-sugar and grape-sugar, both aerobically and anaerobically, and he pointed out the prominence of lactic acid among the products of fermentation.

A decade later Theobald Smith (150) suggested a biological division of *E. coli* when he redefined *B. coli-communis*[4] stating it could be divided into alpha and beta varieties based on sucrose fermentation. Smith is usually credited with pioneer work in determining the gas ratios produced in the fermentations. The results were corrected by later workers using more delicate methods; but it is curious that this promising field has not been exploited more thoroughly. Durham (39) named the sucrose-fermenting variety of the colon bacillus *Bacillus coli-communior*. Among the other species of coliform bacteria early described and frequently encountered in the literature may be mentioned the "Milchsäurebacterium" of Hueppe (1884) described in 1885 by Zopf as *Bacterium acidi-lactici*, and Flügge's *B. neapolitanum* (1885). These two species and *B. communior* appear in Bergey (12) as varieties of *Escherichia coli*.

MacConkey (96, 97) first placed classification of coliform organisms on a comprehensive, biochemical basis. He established

[4] In the review of a reference its terminology is used even if outdated or incorrect as some are.

four primary groups of the lactose fermenters based on the fermentation of sucrose and dulcitol. These groups were further subdivided on the basis of tests for motility, indole production, gelatin liquefaction, the Voges-Proskauer reaction and the dissimilation of adonitol, inositol and inulin. MacConkey's scheme called for 128 types of which he actually isolated 36. By 1928 other workers had described 35 additional types, making a total of 71 to be recognized by that date (133). Climaxing taxonomic work of the MacConkey type, Mackie (100) studied the coliform bacteria of feces and urine, forming four principal groups on the basis of gas production, indole production and inositol fermentation. Subdivisions of these groups allowed for many types of which 73 were found. This contribution is of value today because of its emphasis on the use of inositol and as a further illustration of the difficulties encountered by taxonomists whose schemes of classification proceed on the 2–4–8–16–32 $\cdots$ or "2^n principle."

As pointed out by Topley and Wilson (170), one important correlation between biochemical activity and natural habitat was early recognized. The *A. aerogenes* type was found to be a relatively infrequent inhabitant of the intestine, but was frequently isolated from certain grasses and from the soil, while *B. coli-communior* and *B. coli-communis* were noted to be typically intestinal parasites (185). This correlation was of practical as well as of theoretical importance. The presence or absence of "*B. coli*" in water supplies, and the relative number of this organism if present, soon came to be recognized as a very valuable indication of the presence and degree of fecal pollution, and it became desirable to differentiate between those types which were of intestinal origin, and those which might occur in unpolluted waters.

Thus, editions of Standard Methods of Water Analysis published in the second decade of this century included a classification in which "the following group reactions indicate the source of the culture with a high degree of probability:" and there followed a classification of "fecal" *B. coli*, "fecal" *B. aerogenes*, *B. aerogenes* probably not fecal, and *B. cloacae* which might or might not be

fecal, based on the methyl-red and Voges-Proskauer reactions, the liquefaction of gelatin, the production of indole and the fermentation of sucrose and adonitol. This material has not appeared in recent editions because no confirmation could be found for the early reports that *A. aerogenes* of intestinal origin fermented adonitol whereas *A. aerogenes* from grains and soil did not. Indeed, as we shall see, the prolonged search for tests which differentiate between fecal and non-fecal coliform bacteria has so far been in vain.

As early as 1912 principles of simplification of coliform classification were beginning to appear. Thus Howe (64) showed that there was no correlation between certain features, such as motility, fermentation of dulcitol and mannitol, indole production and nitrate reduction; and he recognized only two species, *B. communis* and *B. communior*. Kligler (79) subdivided the lactose fermenters according to their sucrose and salicin reactions rather than by sucrose and dulcitol reactions, as proposed by MacConkey, since sucrose-salicin sub-groups correlated more highly with the indole, Voges-Proskauer and gelatin reactions than did the sucrose-dulcitol subgroups. The chief emphasis in coliform taxonomy on simplification through the application of statistical methods of correlation of related characters has been expressed by Levine (89, 90). His classification also was one of the first to make use of the Voges-Proskauer test as the primary feature of division of the strains studied.

From quite another point of view Clemesha (28) grouped coliform bacteria according to the resistance shown toward storage. This work represents the most important early contribution to the ecology of the coliform bacteria, possibly of any group, and treats in detail such problems as viability, competition and succession.

Although the first edition of Bergey's Manual of Determinative Bacteriology (1923) allocated 22 species to the genus *Escherichia*, six to *Aerobacter*, and six to *Encapsulatus* (*Klebsiella*) classified mainly on the basis of reactions with fermentable substances, ground had been broken for a somewhat different and more basic approach to taxonomy. The Committee of the Society of Amer-

ican Bacteriologists on the Characterization and Classification of Bacterial Types, appointed at the 1915 annual meeting at Urbana, projected, among other tasks, a study of the colon-typhoid group of bacteria, "a group which, together with certain sharply defined species, includes many puzzling intermediate forms, difficult of classification and yet of fundamental medical and sanitary importance" (186). Out of the interest of this committee, two sets of data emerged dealing with coliform bacteria.

These have been summarized by Yale[5] as follows: "During the period 1914 to 1921, Rogers and associates published a series of outstanding papers on the characteristics and distribution of the coliform group. An especially important contribution was the separation of the group into a low ratio section in which the CO_2:H_2 ratio was approximately 1:1 and a high ratio group in which it was approximately 2:1. In a report summarizing these studies in 1921, Rogers made this statement, 'So far as data are available, the low ratio or *B. coli* group appears to be a very definite and circumscribed entity and there is no apparent reason for separating it into species.' In addition he recognized *B. aerogenes* and *B. cloacae* as separate species in the high ratio group."

"Winslow, Kligler and Rothberg (186) made extensive classification studies and decided that in the low ratio group, only four species were justified (*Bacterium coli, B. communior, B. acidilactici* and *B. neapolitanus*). In the high ratio group, two species were accepted (*Bacterium aerogenes* and *B. cloacae*)."

The methyl-red test (27) was a logical outcome of work in Rogers' laboratory on the fundamental nature of the fermentation of glucose by coliform bacteria. These Washington workers found that the low gas-ratio *E. coli* section produces under suitable conditions a lower pH in glucose broth than do the high gas-ratio *A. aerogenes* organisms. As a result, the former give a red color to methyl red (+) and the latter a yellow color (−)

[5] Round Table Discussion on the coliform group of bacteria, 39th annual meeting, Society of American Bacteriologists, Washington, D. C., December 28, 1938.

in the glucose broth. The formation of acetyl-methyl-carbinol, demonstrable by the Voges-Proskauer test, occurs when *A. aerogenes* is grown in glucose broth but does not occur with *E. coli*. This inverse correlation (M.R.+V.P.− and M.R.−V.P.+) was emphasized by Levine (88). For some years it was held to be well-nigh perfect, but it is now known that coliform organisms occur which are positive to both tests and also those which are negative to both. Thus Stuart and co-workers (161) found that almost ten per cent of their coliform cultures did not correlate.

Another important development has been the recognition of "intermediates," organisms resembling both *E. coli* and *A. aerogenes* but identical with neither. In a study of the utilization of simple nitrogenous compounds, Koser (82) observed that *A. aerogenes* was able to multiply and grow luxuriantly in a medium containing uric acid as the only source of nitrogen. In this medium *E. coli* failed to develop. Later work by Koser (83) developed another, and better, utilization test, i.e., growth in a medium in which the only available carbon source is the citrate radical.[6] In this medium *A. aerogenes* develops very well, whereas *E. coli* fails to grow *noticeably* (140). It was soon found that some methyl-red-positive organisms, presumably *E. coli*, grew as well in the citrate medium as *A. aerogenes* (85). For a time such organisms were spoken of as "soil coli" and there was again revived the hope that sanitarians had a test which would set off fecal from non-fecal organisms. Such organisms are now known as "intermediates" and are found in feces as well as in man's environment.

Werkman and Gillen proposed the genus *Citrobacter* for coliform bacteria producing trimethylene glycol, and described seven species (180). Since such bacteria utilize citrate and are methyl-red-positive, it was thought that "*coli-aerogenes* intermediates" might be allocated to the genus *Citrobacter*, but the genus has not

[6] Pesch (1921) evidently somewhat anticipated the work of Koser on citrate utilization but apparently did not follow it up with further investigations, and he dealt more primarily with other members of the colon-typhoid group than the coliform bacteria.

been recognized by the leading taxonomists. Tittsler and Sandholzer (165), and Carpenter and Fulton (25), favor classifying "intermediates" with *Escherichia* and *Aerobacter*; Minkewitsch (106) calls them *B. coli-citrovorum* Koser; and Parr (123) suggests that they be made a species in the genus *Bacterium*, which would include also *B. coli*, *B. aerogenes* and *B. cloacae*. The fifth edition of Bergey's Manual designates them as *Escherichia freundii*.

In an analysis of data presented in recent papers (1924–1937) dealing with coliform bacteria, Parr (123) found that the reactions earlier used for classification, such as the fermentation of sucrose, dulcitol, salicin, and raffinose, have largely been replaced by other tests. If this analysis be brought down to date it will be found that in 22 of the most recent projects the following tests have been used to establish coliform classification:

	times
Indole production	15
Methyl-red reaction	20
Voges-Proskauer reaction	22
Citrate utilization	20
Uric acid utilization	6
Cellobiose fermentation	4
Gelatin liquefaction	3
Eijkman test	2
H_2S production	2
Sucrose fermentation	1
Inositol fermentation	1
Alpha-methyl-d-glucoside fermentation	1

There is, therefore, justification for the creation of the "Imvic" quartet of tests (119). Imvic is a mnemonic which fixes in order the four tests now in greatest use in classifying coliform bacteria: (I) indole production; (M) methyl-red reaction; (V) Voges-Proskauer test; and (C) the utilization of citrate as a sole carbon source. Four characters give 16 possible combinations. Three of these (+ + − −, − + − −, + − − −) Parr called the *E. coli* section, three (− − + +, − − + −, − − − +) the *A. aerogenes* section, and ten (+ + + −, + + − +, − + − +, + − + −, − − − −, + − − +, − + + −, + + + +,

+ − + +, − + + +) constitute the "intermediate" section, and he stated that 14 of the 16 types have been reported.

Recently, Stuart and co-workers (161) used the Imvic group of tests plus the fermentation of cellobiose in the study of a large series of coliform bacteria. Cellobiose may be fermented with acid production or with the production of both acid and gas, or it may not be fermented at all. Hence, recording three possibilities for cellobiose brings the possibilities of the Stuart scheme to 48 types of which the Brown University group found 21 among the 3247 cultures studied. These could be assigned to nine of the 16 Imvic types. Of the 48 Stuart types Parr has found 16, Skinner and Brudnoy (149) 14, and Oeser (115) 13.

Malcolm (103) classified 1636 coliform strains isolated from milk and bovine feces into eight groups on the basis of the Voges-Proskauer test, citrate utilization, inositol fermentation and indole production. He called one group *B. coli*, three groups "intermediates," one *B. friedländeri*, one *B. cloacae*, one *B. oxytocus*, and one *B. aerogenes*, and he insisted that all should belong to one genus. In addition he encountered 39 anomalous strains which did not fit into his scheme of classification. This work illustrates again the difficulty of classification in this field. The group is so complex and intergrading that each form recovered cannot be assigned an exact name without making the number of such names well-nigh legion. It is refreshing, therefore, to find that Yale in the fifth edition of Bergey's Manual has kept the number of species small, listing some forms as varieties and disregarding scores of names that have confused workers down to the present day.

That the field of biochemical classification has been by no means exhausted is shown by Mitchell and Levine (109), who studied coliform bacteria to determine if nitrogen utilization was as distinctive for differentiation as carbohydrate dissimilation is thought to be. Nucleic acid and its degradation products were employed as nitrogen sources in synthetic media with glucose as the carbohydrate source and indicator. Over 350 strains were tested with yeast nucleic acid, uric acid, allantoin, hydantoin, uracil, urea, adenine and xanthine. Organisms giving positive

reactions with yeast nucleic acid, uric acid, allantoin and hydantoin correlated with the positive Voges-Proskauer test and formed the section *Aerobacter*. *Escherichia* and the "intermediates" were negative to these tests[7] but among them *Escherichia* utilized uracil and failed to utilize urea, whereas the "intermediates" metabolized urea but could not break down uracil. Since both *Escherichia* and *Aerogenes* can utilize uracil and the "intermediates" cannot, Mitchell and Levine feel that they have additional evidence that the "intermediates" should constitute a separate genus. Other evidence is that many "intermediates" produce hydrogen sulphide and those tested yield trimethylene glycol in a suitable medium.

Returning to the Friedländer group it is to be noted that these encapsulated coliform bacteria have not been satisfactorily classified on any such basis as has been used for the other members of the group. Perkins (125) stated that organisms of this group which show no fermentative power are probably degenerate rather than definite entities and can in many cases be reactivated to their original type. Fitzgerald (46) studied 44 cultures of *Bacillus mucosus-capsulatus* and found satisfactory biochemical classification difficult. He believed that mutations, based on the necessity of maintaining a parasitic existence, have caused gram-negative bacilli, found normally in the body elsewhere than in the intestinal tract, to develop capsules for protection, and a new group has arisen designated as *B. capsulatus-mucosus*, connected by the varieties *B. aerogenes* and *B. acidi-lactici* with the non-encapsulated gram-negative bacilli belonging to the colon group.

Winslow, Kligler and Rothberg (186) in commenting on the encapsulated pathogenic forms, said: "It seems evident, either that we are dealing with an extraordinarily variable group, or that forms which are not really related have been identified as of this type merely because of possession of capsule." It should be recalled that *Escherichia coli* not infrequently occurs heavily encapsulated (151, 118).

[7] Parr (123) maintains that some "intermediates" are Voges-Proskauer positive.

THE TRIBE ESCHERICHEAE AND RELATED FORMS ACCORDING TO BERGEY

In the fifth edition of Bergey's Manual of Determinative Bacteriology (12)[8] Family X. *Enterobacteriaceae* Rahn is divided into five tribes, viz.,

Tribe I. *Escherichieae:* 3 genera, 10 species.
Tribe II. *Erwineae:* 1 genus, 13 species.
Tribe III. *Serrateae:* 1 genus, 6 species.
Tribe IV. *Proteae:* 1 genus, 8 species.
Tribe V. *Salmonelleae:* 3 genera, 65 species.

The three genera of the tribe *Salmonelleae* are *Salmonella* with 37 species and 12 additional varieties, *Eberthella* with 14 species, and *Shigella* with 14 species. With the exception of *Proteus morgani*, the coliform group is found in and includes all of the tribe *Escherichieae* which we shall next consider.

Escherichieae trib. nov.—ferment dextrose and lactose with the formation of acid and visible gas. In only one genus, *Aerobacter*, is gelatin liquefied and that but slowly.

"Genus I. *Escherichia.*—Methyl red test positive. Voges-Proskauer test negative. Citric acid may or may not be used as sole source of carbon.

I. Citric acid not utilized as sole source of carbon.
  A. Hydrogen sulphide not produced.
    1. *Escherichia coli.*
II. Citric acid utilized as sole source of carbon.
  A. Hydrogen sulphide produced.
    2. *Escherichia freundii.*

Genus II. *Aerobacter.*—Methyl red test negative. Voges-Proskauer test positive. Citric acid used as sole source of carbon.

I. Glycerol fermented with acid and gas.
  A. Gelatin not liquefied.
    1. *Aerobacter aerogenes.*
II. Glycerol not fermented with acid and gas.
  A. Gelatin liquefied.
    2. *Aerobacter cloacae.*

Genus III. *Klebsiella.*—Methyl red test usually positive. Voges-Proskauer test usually negative. Citric acid usually (?) used as sole

[8] For both galley and page proofs of the new Bergey Manual dealing with the family *Enterobacteriaceae* I am indebted to Dr. Robert S. Breed.

source of carbon. Capsulated forms from respiratory and other mucous membrane regions.

I. Litmus milk acid, but not coagulated.
  A. No acid and gas from maltose or mannitol.
    1. *Klebsiella pneumoniae.*
  B. Acid and gas from maltose and mannitol.
    2. *Klebsiella ozaenae.*

II. Litmus milk acid and coagulated.
    3. *Klebsiella granulomatis.*
    4. *Klebsiella capsulata.*
    5. *Klebsiella paralytica.*

III. Litmus milk unchanged.
    6. *Klebsiella rhinoscleromatis.*"[9]

OCCURRENCE AND SIGNIFICANCE OF COLIFORM BACTERIA, INCLUDING PATHOLOGY

In the discussion which follows an effort will be made to present and preserve the ecological point of view which should help to explain why encapsulated coliform bacteria occur in one environment and non-encapsulated in another. A shift of environment may provide opportunity for the development in large numbers of species which survive in small numbers and with difficulty in another milieu. Or, if we are dealing with pure cultures, changes in environment may favor the survival of the descendants of one variant or mutant over those of another. Natural selection has probably not caused organic evolution but it is one of the most important factors in its direction. What happens to an association of bacteria or to a pure culture of a single organism with capacity for variation or mutation will in large part depend on the environment.

The entire family *Enterobacteriaceae* Rahn, with the exception of the plant parasites, and the red pigment-producing chromogens, are thought of as intestinal bacteria, having, however, in the case of the more saprophytic species a considerable distribution outside the body of man and animal in nature.

Animal pathology. Very little attention was paid to the coliform group in veterinary medicine until comparatively recently.

[9] From page proof, fifth edition, (12), Manual of Determinative Bacteriology.

Citations include *Proteus morgani* infections of zoo animals, gaseous emphysema, abortion in sheep, a disease of white rats associated with parasitic infestation, "moose disease," diarrhea in foals and in young pigeons, calf "scours," infectious enteritis of young lambs, a fatal disease of carp, metritis of mares, pneumonia in beavers, mastitis in cows, infectious diarrhea of chicks and abortion in ewes.

TenBroeck recently stated (1938) that it is extremely difficult to evaluate the importance of the colon group in animal pathology but that they take the place of the streptococci in man, i.e., they are often secondary invaders that complicate infection. Plastridge (1938) involved coliform organisms as responsible for navel ill in chicks, navel ill in calves, and a limited number of acute cases of mastitis in cows, and Hitchner (1938) stated that slow lactose-fermenting coliform bacteria have caused several epidemics of intestinal disease among chicks in Maine.

It will be noted that coliform bacteria produce "scours" in calves, diarrhea in foals and pigeons, infectious enteritis in young lambs, and infectious diarrhea in chicks. This pathogenicity for the young of animals is significant. Calf "scours" has been the subject of a classical investigation by the Rockefeller group at Princeton. The coliform bacteria concerned are usually in the mucoid phase (151, 95) but they may not be (Dollahite, 1938). Certain points are significant. The disease is produced by a soluble, diffusible substance of exotoxic nature (153, 131). This toxin is a capillary toxin and is far less effective in the usual laboratory animal than in the calf. Colostrum or maternal serum will protect the calf. The disease is one of intoxication and diarrhea with resultant dehydration. "If we put all the facts together the inference seems admissible that scours is associated with special races of *Bacillus coli* and that such races are developed and maintained in large herds. This will account for the different races of *Bacillus coli* which have been charged by other observers as the cause of scours. Each large herd through the presence of calves below par at birth may thus develop and maintain its own type of scours organism which, however, is not virulent enough to make any headway in naturally

strong calves properly cared for as regards food and housing" (Smith and Orcutt, 151). Somewhat the same view is held by Lovell (131) who concluded that special races of *Bact. coli* pathogenic for young calves exist, but that more than one race may be present in a herd and sometimes more than one type may be isolated from an individual calf.

The importance of this disease may be inferred from the statement of Dollahite (1938), released by Schoening (1938), that during the first five months of 1938 in a large government-owned dairy 68 calves were born, of which 33 (49 per cent) died with acute dysentery before they were five days old. Many of them died within 48 hours after birth.

In this outbreak *Escherichia communior*, *E. acidi-lactici*, and rarely *E. coli*, were encountered with *E. communior* occurring in about 60 per cent of the cases. From June 15 to November 15, 1938, cow serum prepared with a pooled *E. communior-E. acidi-lactici* antigen was given intravenously twice on successive days to 35 new-born calves with the result that none of the calves died, whereas of 32 others born during the same period and left untreated, ten died of acute dysentery before they were seven days old.

The coliform strains most concerned in animal disease are *E. coli*, often in the mucoid phase and often atypical as to lactose fermentation, and members of the genus *Klebsiella*.

Human pathology. *Proteus morgani* has been reported as the etiological agent in summer diarrhea of infants, infectious diarrhea of the new-born, diarrhea and dysentery in adults, infections of the urinary tract, meningitis, chronic discharging wounds, ulcerative colitis, war wounds, fatal septicemia and a paratyphoid-like infection. Rauss (130) placed Morgan's bacillus with *Proteus*, the British System of Bacteriology (1929) put it in the dysentery group, and Winslow, Kligler and Rothberg (186) considered it a paratyphoid (*Salmonella*) which was the classification given it in the third edition of Bergey (1930), an allocation approved by Levine, Ajwani and Wedin (91), Havens and Mayfield (57), and sanctioned by the French Dictionary (56). Thjøtta (164), d'Aunoy (3), Waaler (177), and Jordan, Crawford

and McBroom (69) think of it as a coliform type because it is gas-producing, actively motile, strongly positive for indole, extremely heterogeneous serologically and distributed in a number of environments, including soil, water and normal stools (80). Since the fermentation of lactose is delayed or even absent, it seems logical to consider the Morgan bacillus as standing with the paracolon bacilli on the border of the coliform group next to the paratyphoid group, *Salmonella.*

Organisms of the genus *Klebsiella* are reported from diseases of the respiratory tract, rhinoscleroma, war wounds, suppuration, meningitis, gaseous emphysema, septicemia, fetid nasal catarrh, infections of the urinary tract, infectious diarrhea of the newborn, and bronchial asthma. The Friedländer type of coliform organism is not prominent in diarrhea, dysentery, cystitis, pyelitis, cholecystitis and cholangitis, nor is it as relatively prevalent in post mortem invasion of the body as many have supposed. Mackennon, Turner and Khayat (98) reported a study on bronchial asthma in which 28 strains of "mucoid encapsulated organisms" were studied. They confirmed the generally accepted view that such strains show very variable cultural reactions and that when present in bronchial asthma there was an associated hypersensitivity of the patients towards the intradermal test with vaccine prepared from the bacilli. The presence of gas in the tissues discovered on autopsy is usually attributed to *Clostridium welchii,* but coliform bacteria can cause this condition.

Coliform organisms not specifically labelled as *Klebsiella* or the Morgan bacillus have been reported from such conditions as pyelitis, cystitis, cholecystitis, cholangitis, suppuration, septicemia, war wounds, Winckel's disease or hemorrhagic septicemia of the new-born, sepsis neonatorum, infectious diarrhea of the new-born, gastro-enteritis, food poisoning, peritonitis, diarrhea, meningitis, arthritis, intestinal intoxication, gaseous emphysema, and rare cases of infectious dermatitis.

The importance of coliform bacteria in cystitis and pyelitis is attested to by a very large literature on the subject. The two points of chief concern to the bacteriologist working in urology are: (1) the mechanism of the invasion of the urinary tract by

bacteria; and (2) the types of coliform bacteria concerned. For two decades Dudgeon and his co-workers represented the most active group in this field (35, 36, 37). Their early work emphasized the property of hemolytic power as characterizing most of the coliform types involved, particularly in the male, and somewhat later they called attention to the slow lactose-fermenting coliform bacteria in urine. Although practically all of the atypical coliform bacteria were first isolated from feces, they were early found in urine. Thus Mair (101) described the paracolon bacillus from urine, W. J. Wilson (183) reported "anaerogenous" coliform bacteria from that source, and Kennedy, Cummings and Morrow had in their series of slow lactose-fermenters four strains from urine (78).

Hill, Seidman, Stadnichenko and Ellis (62) made an exhaustive report on the coliform bacteria isolated from cases of genito-urinary infection. They classified 200 cultures into *Escherichia*, 50 per cent; *Aerobacter*, 39.5 per cent; *Proteus*, 2.5 per cent; and miscellaneous, 8 per cent. Their data permit a breakdown of the 179 coliform strains (89.5 per cent) into *Escherichia*, 27 per cent; "coliform intermediates," 23 per cent; and *Aerobacter*, 39.5 per cent. More than half the strains were hemolytic.

Burke-Gaffney (19) studied 1000 strains of coliform organisms isolated from 126 specimens of urine. Classified by MacConkey groups 18 per cent were *B. acidi-lactici*; 7 per cent, *B. coli-communis*; 27 per cent, *B. coli-communior*; and 48 per cent, *A. aerogenes*. On the basis of indole production, methyl-red reaction and citrate utilization, 33 per cent were *E. coli*, 52 per cent *A. aerogenes*, 10 per cent "intermediates," and 5 per cent "atypical." Sandholzer (142) reported on 530 cultures of coliform bacteria isolated from 283 patients with urinary infection. Of the 530 strains 83 per cent were *Escherichia* belonging to 27 species or types, and 13 per cent were *Aerobacter* belonging to 14 species or types. The relative abundance of *Aerobacter* strains in urinary infections is striking. Hill et al. (62) reviewed the literature on the prevalence of *Aerobacter* in feces and found that among 14 reports totalling nearly 7000 cultures there were five reporting no *Aerobacter*; and in the other nine the percentage

occurrence of this genus ranged from 0.06 to 16.0 per cent. Their own data showed 39.5 per cent of *Aerobacter* in urological infections, and they stated that if the source of such infection is intestinal it is possible that the fecal organisms finding their way into the urine respond to some selective action in the genito-urinary tract which operates to favor the genus *Aerobacter* over *Escherichia* since in the bowel *Aerobacter* is far outnumbered by *Escherichia*. This point is well supported by the data of Burke-Gaffney and to a lesser degree by those of Sandholzer. If the analysis be made on the basis of citrate utilization the difference is even more marked. Ruchhoft, Kallas, Chinn and Coulter (140) summarized the findings of six workers on 2534 coliform cultures from feces. Only 9.2 per cent of these were citrate-positive. In the Hill series from urine there were 62.5 per cent of citrate utilizers. Here is indeed a nice example of the operation of ecological factors.

Food poisoning is not thought of as of coliform etiology but Buchanan and Megrail (17) in Ohio, and Gilbert, Coleman and Laviano (51) in New York have reported outbreaks apparently due to organisms of the genus *Aerobacter*. If coliform organisms can produce a toxin, as seems amply demonstrated, it is a little odd that more intoxications with this toxin have not occurred. It may well be that the human adult is relatively resistant to it.

In recent years considerable interest has been aroused by the occurrence of outbreaks of epidemic diarrhea and gastro-enteritis, apparently water-borne, but in which no definite pathogen as etiological agent can be demonstrated (176, 181, 50, 188). This problem has assumed such proportions that a special symposium on gastro-enteritis was held by the American Water Works Association in connection with its annual meeting at Buffalo in 1937 (29).

Infectious diarrhea of the new-born is a disease in which emphasis should be placed on the coliform group as probable etiological agents. This disease is highly fatal and infectious in nature and when it invades a hospital nursery it is sometimes brought under control only by closing the maternity service of the institution. The nature of the disease indicates a potent intoxica-

tion which induces a diarrhea and results in extreme dehydration. Its analogy to the clinical course of Asiatic cholera has occurred to some of the students of the problem and comparative pathology calls to mind the calf scours situation. Among recorded outbreaks Dick, Dick and Williams (33) reported *Proteus morgani*, Jampolis, Howell, Calvin and Leventhal (68), a form of *Klebsiella*; Dulaney and Michelson (38) found *B. coli-mutabile*; McKinlay (99) recovered an organism thought at first to be a paratyphoid but which was probably a coliform organism of paracolon type; and Randall (1938) encountered coliform organisms in his study of two cases.

Theobald Smith's philosophy concerning *E. coli* is pertinent at this point. He stated (152) that in the gradual evolution of pathogenic or invasive types of bacteria, the beginnings of parasitism may have been made possible by a soluble diffusible toxin, but that in later stages this primary offensive, more or less accidental, mechanism is either partly or wholly suppressed and some different mechanism developed with which the bacteria protect themselves against the body-foreign forces of the host. The process may be regarded as shifting from the destructive, predatory to the parasitic, from the offensive to the defensive type. According to this hypothesis, *E. coli* represents the early predatory toxic stage with, however, a certain specialization towards protection from anti-foreign activities in the digestive tract. It represents in many respects the cholera vibrio in its activities.

In a recent discussion on staphylococci (Levine, B. S., 1938) it was stated that probably all of the *Staphylococcus aureus* type possess the capacity to produce exotoxin. Only certain strains produce enough to induce food poisoning in man when ingested in cream puffs or other food. There is evidence that somewhat the same situation may hold among coliform bacteria.

In all considerations of gastro-intestinal disease of infectious nature one should not lose sight of the possibility that symptoms are being manifested in the bowel whereas the inciting cause is elsewhere in the body. Felsen (45) stated that the indirect hematogenous excretory mechanism of the intestine is important in explaining many poorly understood, non-specific intestinal

infections or so-called infectious diarrheas. The primary cause often exists outside the intestine, and search for specific noxious agents in the bowel is then futile. Focal intestinal symptoms often cease abruptly after the primary extra-enteric focus of infection is eliminated, but they may persist for a longer period if necrosis and ulceration have been produced. The possible rôle of upper respiratory infection on the bacteriology of the intestinal tract has recently been discussed by Lieb and Chapman (94). In cases where the intestinal manifestations are incited extraneously the bacteria of the bowel may well respond to the new conditions with an altered flora which might serve as an "indicator" (121). Sufficiently studied, such bacterial types might be resolved into instruments of diagnosis almost as surely as if they were of primary etiological significance. We have then to study infectious diarrhea of the new-born either as a locally incited disease produced in a susceptible host by toxin-producing coliform bacteria, or to consider it as of other etiology, probably viral, with the avenue of infection by way of the respiratory tract.

Plant pathology. In this connection it is desired merely to emphasize that the recognized coliform bacteria and the 13 species of *Erwinia*, listed in Bergey, are very closely related. F. D. Chester (1938) stated that the genera *Erwinia* and *Phytomonas* were established on a purely utilitarian basis and have no genetic standing. Stanley (158) was of the opinon that the soft rot bacteria undoubtedly belong to the colon-typhoid-dysentery group of bacteria. Stuart, Griffin and Baker (161) studied 200 "coliform" cultures obtained from decayed portions of a number of fruits and vegetables. Serological investigations, in progress, seem to show an antigenic relationship between the plant, atypical and typical coliform organisms.

Occurrence in the intestine. Coliform organisms (*Escherichia* and *Aerobacter*) were first isolated from the intestinal tract of man. They were shortly recognized, though not without considerable research, as occurring in the intestinal tract of all higher animals. In examinations of meconium, commonly considered as sterile, it has been shown (22, 55, 155) that coliform

organisms may be present in a certain percentage of specimens. Throughout life, man is rarely without demonstrable coliform bacteria in his gastro-intestinal tract.

Much research has been expended in the effort to discover tests which will select fecal from non-fecal coliform bacteria (149, 18, 140, 6, 25, 122, 123, 103, 161). Certain facts emerge from this mass of data. All types of coliform bacteria may occur in feces but *Escherichia coli* (Imvic + + − −) is the most typical, numerous and constant type, with *Aerobacter aerogenes* (− − + +) next, and coliform intermediates (− + − + most common type) third. *A. cloacae*, paracoli, slow-fermenters, and *Klebsiella* may also be recovered. There is not much point in making comparisons of data unless identical methods of isolation have been used. In a certain number of cases *E. coli* may be absent, and there are even fecal specimens which yield no coliform bacteria at all (25, 123). Furthermore the coliform flora of an adult in good health and on a constant diet may show considerable change from day to day (122). When the usual fecal specimen is stored in saline suspension in the ice box considerable change occurs in the coliform flora, *E. coli* decreasing, and "intermediates" and *A. aerogenes* and *A. cloacae* increasing with, after many months, a complete change in flora often ending up with slow lactose-fermenting varieties of citrate utilizers. In about 14 per cent, however, there is no such change, the original *E. coli* persisting for months in competition with the other fecal bacteria and still presenting the characteristics of *E. coli* from fresh feces. These data are interpreted by Parr (119) to mean that in the latter case the specimens were originally pure cultures of *E. coli* so far as coliform bacteria are concerned.

The significance of these findings for sanitary science is that all of the coliform bacteria must be thought of as possibly fecal in origin. Where pollution derives from several sources one may expect to find *Escherichia coli* if the pollution be fresh; where pollution is from a single source there is no certainty that *E. coli* will be present; and the finding of typical *E. coli* may not indicate fresh pollution, particularly if that pollution be derived from a single source. Despite these qualifications, the presence of

significant numbers of *E. coli* in water remains our best test for fecal pollution.

Occurrence in milk. The recent literature on coliform bacteria in milk is even more voluminous than that for these organisms in feces (81, 44, 102, 182, 6, 7, 187, 10, 115, 162). Stark[10] has stated that, due to what we may call the "living conditions of cows," most raw milk contains coliform bacteria. These organisms in milk are assumed to come from barnyard manure, and since cows do not have typhoid fever, the presence of coliform bacteria in raw milk is known not to be of the same public health significance as is their presence in water. They are uniformly regarded as undesirable bacteria to have in milk and dairy products because they produce gas and undesirable flavors and odors. Their significance is largely proportional to the numbers present. It is important to remember that, unless some inhibiting condition is present, these bacteria grow well in milk. Although bacteria belonging to this group are occasionally found able to resist the heat treatment of the pasteurizing process, their presence in pasteurized milk is usually interpreted to indicate recontamination. The seriousness of permitting pasteurized milk to become recontaminated with any kind of bacteria is readily recognized. The pasteurizing processes applied to cream for buttermaking and ice cream mixes are generally accepted as adequate to destroy coliform bacteria. Their presence in these products is also believed to indicate recontamination of a pasteurized product. The types of coliform bacteria present in milk will vary with the flora of the feces, soil, or grain dust contaminating it.

Occurrence in soil. Coliform organisms are common in soil. Minkewitsch, Rabinowitsch and Joffe (108) believe that these bacteria are not found in the soil where there is no animal life. As pointed out by Thom (1938), it has been assumed that the presence of the colon group in soil is due to fecal contamination, and for that reason coliform bacteria have not particularly engaged the attention of soil microbiologists. In soil *A. aerogenes* is more abundant than *E. coli*, and "intermediates" and atypical

[10] Coliform Round Table, 1937.

forms are present. This picture will vary with the character and use of the soil from the *E. coli*-sparse, virgin, protected soil to the *E. coli*-rich pasture grazed over by animals. From some quarters there is evidence that some, at least, of the citrate utilizers and atypical forms in soil are derived from fecal *E. coli* and typical intestinal forms. Minkewitsch, Rabinowitsch and Joffe (108) report the change of *E. coli* seeded in soil into citrate-utilizing "intermediates." This is far from the production of *A. aerogenes* from *E. coli* which, so far as we are aware, has never been reported, but it does indicate a step in coliform evolution. Parr (124), working with one of Koser's original soil strains, V5, in laboratory cultivation for more than a decade, has derived *E. coli* (+ + − −) from the strain called originally an "intermediate" (+ + − +). Despite the fact that citrate utilizers predominate there is evidence that *E. coli* can survive for a considerable time in soil (121).

Occurrence in urine. The striking thing in urine, as in soil, is the shift in the coliform picture from what it is in fresh feces to a predominance of citrate-positive coliform bacteria (62, 19, 142). The same shift occurs, as we have shown, when the usual fecal specimen is stored. The mechanism of these shifts may be a matter of variation, but is more likely succession, conditioned by ecological factors.

Occurrence in water. The literature of water bacteriology is much too complex to be reviewed here, as it touches on mediums, tests, interpretations and standards (139, 140, 18, 52, 169, 24, 48, 8, 60, 128, 14). Personal communications (1938) from Kulp, Levine, Norton, Butterfield, Mickle, McCrady, Norcum and G. F. Edwards have called attention to many sanitary problems connected with the coliform bacteria. Kulp feels that an attempt should be made to differentiate between *E. coli* and *A. aerogenes* especially when dealing with private water supplies, whereas Butterfield states that it is his policy to attach equal sanitary importance to the presence of each member of the coliform group since all are found in feces and since they are about equally susceptible to the forces of natural and artificial purification processes. Norcum is concerned over the increasing prevalence

of gastro-enteritis, apparently water-borne, but with questioned etiology. Norton mentions the significance of the work of Heathman, Pierce and Kabler (60) from which it appears that *E. coli* may be no more (or even less) resistant to chlorine than the typhoid bacillus, and he feels the chlorine resistance of coliform bacteria should be restudied. McCrady is also anxious to have the significance of atypical coliform bacteria cleared up, and the New England workers are particularly concerned over the necessity for generous interpretations of standards to avoid condemning too many water sources epidemiologically satisfactory. Coliform bacteria do not occur ordinarily in water except from contamination with soil washings and fecal material from man and animal. When the pollution is from feces these bacteria survive for some time but generally with a shift from citrate-negative predominance to citrate-positive predominance. There are, however, both theoretical and actual conditions under which *E. coli* may persist with typical reactions for long periods of time. Usually, though, the numbers of coliform bacteria decrease and in the absence of recontamination the group is usually lost sight of after a few weeks.

"Pump infection," and paper and wood pulp. It is well established that coliform bacteria grow well on leathers and other organic pump-parts, on swimming-pool ropes, and in pipe slime (24, 87, 1, 144). The growth of these bacteria in water distribution systems, of course, affects the analysis of the water. L. S. Stuart (1938) has reviewed the bacteriology of the tanning process from which it is apparent that modern leather is not itself the source of these coliform growths. The forms which are likely to occur naturally are "intermediates" and *A. aerogenes*, but other organisms will grow on jute and leather (*Serratia*, *Escherichia*, and even the typhoid bacillus).

The part played in the paper and wood pulp industries by coliform bacteria is but seldom mentioned. Tonney and Noble (168) have noted the persistence of *E. coli* and *A. aerogenes* on wood. The quality of water in contact with wood may be impaired by a high coliform count under conditions somewhat analogous to pump "infection." In 1931 Beckwith (11) re-

ported on the bacteriology of pulp slime and pointed out the importance of *A. aerogenes*. In 1938 he stated that it was his opinion that pulp slime has as one of its important causes the growth of capsular bacteria, nearly all of which are coliform. He showed, with Morgan, that the mucoid type appears frequently if the incubation temperature is low, and, of course, in the presence of a certain amount of carbohydrate. In "white water" the temperature is low, and it frequently contains appreciable amounts of carbohydrate which possibly are produced by inversion of the cellulose. Sanborn (1938) indicated the need for a detailed study of the coliform organisms found in pulp and paper mill systems and stated that he had seen pulp wood logs coated with gelatinous slime due to the development of organisms related to the genus *Aerobacter*. Chlorination and the high temperature of drying eliminate the bacteria so there is but little danger of the spread of bacteria by paper containers; but pulp containing slime organisms works up into defective finished products so that the problem is one of economic importance.

Olives. Alvarez (2) studied the blister-covered olives commonly called "floaters" and found that the condition was caused by atypical organisms "closely allied to, but not identical with the colon group." One strain, "H," resisted 80°C. for 45 minutes. Ten per cent salt solution was required to kill it in 24 hours. Again, Tracy (171) emphasized the spoilage of olives by colon bacilli. The reviewer has been given to understand that the coliform group constitute the most important olive spoilage organisms and that recoveries are mostly "intermediates" and *Aerobacter*, but occasionally *Escherichia*.

Shellfish. The presence or absence of fecal pollution in oysters and mussels is determined by examination for coliform bacteria (13, 126). In 1938, Perry stated that the examination of shellfish and their growing waters cannot be considered in the same category with drinking water which can be filtered, chlorinated or protected. Perry holds that many coliform bacteria, particularly of the *E. cloacae* type, are present in shucked market oysters or shell oysters when the temperature exceeds 60°F., that they are without significance as indicating pollution, and

that *E. coli* is the logical indicator of fecal pollution in shellfish and shellfish growing waters.

Foodstuffs; miscellaneous. It would appear from the nature of the processing procedures involved that canned foods do not contain coliform bacteria, a surmise confirmed by Williams (1938). Crossley (30) found 88 per cent of 14,365 samples of meat and fish pastes sterile, with coliform bacteria having small significance among the positives. In other types of foods their importance is greater as shown by the report of Griffiths and Fuller (53) on the detection and significance of *E. coli* in commercial fish and fillets, and that of Hunter (65) who found coliform bacteria important in salmon spoilage. One of the "believe it or nots" of bacteriology is the record of Simonds (148) that in a World War depot in Belgium three barrels of soft soap exploded due to growth of bacteria of the genus *Klebsiella* in the soap. The work of Burkey (20) on the fermentation of corn stalks and their constituents by bacteria of the genus *Aerobacter* has further extended our appreciation of the ubiquity of coliform bacteria. Lastly, Minkewitsch (107) has pointed out the part that insects play in the spread of coliform bacteria in the soil and on plants.

ATYPICAL COLIFORM BACTERIA

The significance of atypical coliform bacteria was early recognized, for in 1899 a committee composed of Veranus A. Moore, J. G. Adami, Elmer G. Horton and J. Monjares, was appointed by the section of bacteriology and chemistry of the American Public Health Association to study variations of the colon bacillus in relation to public health.

For convenience we may divide the atypical coliform bacteria into two classes. There are, first, those forms which give most of the reactions peculiar to a particular species but differ from it in some slight degree not sufficient to be named as another species. Such, for instance, are chromogenic *E. coli* (116, 120, 167, 161); encapsulated *E. coli* (153, 118); the sugar-tolerant coliform organism described by James (67); the organism giving common

serological reactions at high titre with the *Salmonella* (54); *A. transcapsulatus* (163), in which the organism lies at right angles to the greatest diameter of the capsule; *E. coli* with polar flagella (66); a heat resistant form (2); the organisms which are methyl-red positive and also Voges-Proskauer positive, or "double negative"; gelatin-liquefying *E. coli;* cellobiose fermenting *E. coli;* and hydrogen-sulfide positive *E. coli.* The property of hemolysis is hardly an atypical feature for it is common to many strains of *E. coli* both from the urine and the bowel. Such atypical forms are confusing to the taxonomist but probably not as much so to sanitarians as the second class of atypical coliform bacteria.

In the second category we place the instances of fermentation irregularities encountered in these bacteria. It will suffice to consider only irregularities encountered with lactose. If one seeds a tube of lactose broth with a typical coliform organism, within 24 hours full acid and gas production will appear. In water analysis, a positive tube must show acid and gas production within 48 hours. What about the tube which has acid and only a bubble of gas in 24 hours but never any more, or one which has full gas production but requires 72 hours to produce it? These are the organisms concerning which McCrady (1938) circularized workers interested in coliform bacteriology.

Moreover, coliform organisms are frequently encountered which fail to ferment lactose for a considerable number of days. Such strains are often confused with paratyphoid bacteria. They are the true slow fermenters and in many cases can be trained to rapid fermentation. Many of them are *Bacterium coli-mutabile* or mutabile types of *Aerobacter* and as such appear to be unstable variants as described by Deskowitz (32), earlier called "mutants."

Other atypical forms are those strains which ferment lactose producing acid but failing to produce gas. For these the term "anaerogenous" is used. Again there are strains which give all of the reactions for *Escherichia* except the fermentation of lactose and which fail to give serological reactions with *Salmonella.*

These are called "paracoli" and may not ferment lactose no matter how long cultured. One further variant is the strain that ferments lactose at room temperature but not at 37°C.

The prevalence of such strains is indicated by Malcolm's work (103) with 1636 cultures of which 3 per cent were atypical. Kline (81) found 126 "anaerogenous" *E. coli* among 325 cultures isolated from raw and pasteurized milk. He expressed the opinion that these organisms are really members of the colon group which may have become modified through the influence of an unfavorable environment. We believe the evidence warrants the view that the slow fermenters, the "anaerogenous" strains, Morgan's bacillus and "paracoli" strains are all coliform bacteria which may be placed with whichever species they have the most characters in common.

It is much more difficult to assess the significance of the slow fermenters and other atypical coliform bacteria. It has been suggested that there is some relationship between the power to ferment lactose and virulence, as shown by the fact that the pathogens of the colon-typhoid group do not ferment lactose, and also by Dudgeon's (37) account of 49 cases of very severe acute infection of the genito-urinary tract in which all the strains of *B. coli* showed delayed fermentation of lactose. It has been found that atypical forms are likely to occur in stool specimens from subjects showing evidences of gastro-intestinal ill health (47). If these points of view be true, it would seem that the atypical strains encountered in water analysis should have more significance as indicators of dangerous pollution from feces or urine than more typical strains. Difficulty arises from the fact that atypical strains are also found in many environments in which coliform bacteria without pathological significance survive. This is known to the water bacteriologist who is inclined to look upon slow lactose-fermenting coliform organisms as "attenuated" or "devitalized" forms.

VARIATION IN THE COLIFORM GROUP

The "unstable variant" is by far the most interesting of bacterial variants. *Bacterium coli-mutabile* (113, 104) is a good

example. When this organism is cultured on lactose indicator-agar it appears not to ferment lactose. After some days, however, papillae appear growing on or out of the original colonies. Sub-cultures from these secondary colonies give typical lactose fermentation but sub-culture from the primary colony, avoiding contact with the papillae, gives delayed fermentation and will, when again plated, reproduce the original picture of colonies, negative to lactose, but on which lactose-fermenting secondaries eventually appear. One may take such a strain and plate it serially hundreds of times. It will still produce non-fermenting colonies on which fermenting papillae later appear. Such strains Deskowitz called "unstable variants." The early workers (113) thought of them as de Vriesian mutations, Stewart (159) attempted to explain them on Mendelian principles, and Mellon (105) has considered *Bacterium coli-mutabile* as a transitional developmental stage between the normal strain of *E. coli* and wild, non-lactose-fermenting *E. coli*. Such "unstable variants" are not uncommon and their peculiar type of variation is manifested in changes in colony type as well as in biochemical reactivity. Thus Deskowitz was working with the R—S colony type variation as manifested by certain strains of *Salmonella aertrycke*; and what appear to be "unstable variant" phenomena are recorded by Koser and Vaughan (86) in their paper on the utilization of d-arabinose by bacteria. It is possible, also, that the citrate "mutant" described by Parr may be another instance of "unstable" variation. It should be stated that except in the case of capsulated forms the discussion deals, so far as the records show, with smooth phase cultures.

Recent work with variations in the ability to ferment sucrose have interested workers in the coliform field and challenged taxonomists. Sherman and Wing (146) found that certain recently isolated strains of *E. coli* and *A. aerogenes*, seeded in pure culture in salicin and sucrose broths, gave rise to progeny which varied from the parent strains used. For example, from a culture of *E. coli*, which fermented salicin but not sucrose, progeny of four fermentative types were obtained which would by some terminologists be named as four different species. Treg-

oning and Poe (172) confirmed the production of sucrose variants, whereas Fulton (49) was unable to do so. Minkewitsch, Rabinowitsch and Joffe (108) have also reported the production of sucrose-fermenting strains from sucrose-negative antecedents. There seems to be marked difference in the facility with which strains of these bacteria vary and the frequency of appearance of strains capable of variation. Thus in our work we encountered up to the summer of 1938 only 29 instances of coliform strains giving the citrate "mutation." But, June 2, a fecal specimen was examined in which 54 of 60 colonies, picked, purified and studied, were *E. coli* which gave off in each instance small numbers of variants that one would have to classify as atypical *E. freundii* since they were citrate-positive and hydrogen-sulfide negative.

Nyberg, Bonsdorff and Kauppi (114) reported that two of their strains changed from *Escherichia* to *Aerobacter*. This statement was made on the basis of a change from M.R.+V.P.− to M.R.−V.P.+ after isolation. Citrate was not used in this work. Such changes were observed by Koser (84) who reported eight soil coliform strains which reversed their methyl-red and Voges-Proskauer reactions. Koser's cultures were, however, all positive utilizers of citrate so that the change observed was not from *Escherichia* to *Aerobacter* but a shift of type within the "intermediate" group. Minkewitsch, Rabinowitsch and Joffe (1936) reported changes *in vitro* and in the soil of *E. coli* to "intermediates." On the basis of their findings they suggested that it might be argued that all coliform bacteria arise from fecal *E. coli*. Most workers, however, seem to feel that the direction of evolution in the coliform group has been from the highly reactive, ubiquitous *A. aerogenes* to the less reactive, more specialized parasitic types.

Passing over numerous interesting references to the alteration of cultural finding in the coliform group through the use of chemicals, immune serum, and the like, we next discuss "shifts." Nyberg, Bonsdorff and Kauppi (114) in 1935 studied 200 cultures isolated at Helsingfors in 1933. They found that 68 strains were not viable and that only 25 of the 132 viable cultures had the

original colony type and cultural reaction. Fifty-nine cultures had changed in both colony type and reaction and 11 more, although retaining the original colony type, gave different reactions. Stuart, Griffin and Baker (161) studied "shifts" in the reactions of 191 cultures and found that in 47 instances changes occurred. They suggest that it might be better to use the term "stabilization" rather than "purification" for the treatment to be accorded cultures, and hold that "purification" implies contamination by a foreign species whereas "stabilization" implies a reasonable constancy of reaction without excluding the possibility of variation under suitable conditions.

SEROLOGY

Van Loghem (173) early emphasized the serological heterogeneity of the coliform group, stating: "Das individuelle Benehmen der Coli-Bazillen bei serologischen Untersuchungen ist bekannt. Stellt man ein Immunserum her mit einem bestimmten Coli-Stamme, dann findet man selten andere Coli-Stamme, welche von diesen Serum agglutiniert werden." Even fecal strains isolated from the same plate will not generally be influenced by the antisera prepared from any of the others. There are two possibilities. Either the number of kinds of *E. coli* is very considerable or the serological variability is very great. Such extreme variability could only be conceived of on some such theory of antigenic flux or instability as van Loghem had in mind: "der Rezeptorenapparat des *B. coli* sich in einem Zustande stätiger immer Verschiebung befindet, so dass sie Characterzüge, welche bei anderen Bakterien die spezifischen Serumreaktionen ermöglicht haben, bei Coli-Bazillen bald wieder ausgewischt werden."

Mackie (100) stated that, while an immune serum to a particular strain of typhoid bacillus will agglutinate most strains of *B. typhosus* with but little variation in degree, immune serum to certain *B. coli* types, on the other hand, have been found to exert little or no action on other strains identical as regards cultural reactions with that used for immunization. Smith (154) said: "The relation between a strain of *B. coli* and its mutant

with reference to the production of agglutinins and protective antibodies may be expressed by the statement that the original strain when injected into cows develops antibodies both toward itself and the mutant, whereas the mutant produces them only towards itself." The citrate "mutant" reported by Parr (124) reacted to the same titre as the parent strain with a serum prepared against the parent. On the other hand, Sievers (147) reported that a coliform strain gave two variants, a gas former and a strain which did not form gas. Sera were prepared from both variants and these sera were not identical. Havens and Irwin (58) also observed an antigenic change coincidental with the acquisition of sucrose fermentation in the Morgan bacillus, "no cross-agglutination" occurring between the sucrose-fermenting and the non-sucrose-fermenting "strains from the same culture."

Lovell (95), employing the precipitin test, found that 79 of 110 strains of coliform bacteria from diseased calves fell into eight groups. Hitchener (1938) prepared sera against eight slow lactose-fermenting strains and tested 19 cultures of these organisms against the eight sera. Four strains were encountered which fell into one group but the others were individualistic. It is true that Dudgeon, Wordley and Bawtree (36) found that most of their hemolytic strains of coliform bacteria isolated from acute urinary infections were agglutinated by a serum prepared from one of them. However, the non-hemolytic cultures from the same source showed no such relationship.

Serological work with coliform bacteria of the genus *Klebsiella* has, however, led to more satisfying results. In 1926 Julianelle (71, 72, 73) established the fact that the specificity of these organisms resides in their capsular materials. He studied a series of Friedländer bacilli and classified them into three specific types A, B, and C, and a heterogeneous group X. Edwards (40) tested 50 strains of encapsulated bacilli and was able to place 43 in two serological groups, seven remaining untyped. In 1929 he found that five cultures of *Bact. aerogenes* were serologically identical (as then tested) with type B Friedländer bacilli and two were identical with a strain of the granuloma bacillus.

In 1934 Morris and Julianelle studied rhinoscleroma strains and found them serologically identical with type C Friedländer bacilli. Barnes and Wight (9) studied a hemolytic, encapsulated strain of *E. coli* which appeared to have antigenic identity with pneumococcus type I. In 1937 Julianelle (75) examined strains of *Bacterium aerogenes* and showed there were three type-specific immunological entities among them, one common to pneumococcus type II, one common to both pneumococcus type II and Friedländer's bacillus type B, and a third which was individualistic. He also showed that strains of *Bacterium aerogenes* differ serologically when encapsulated, but become antigenically the same when de-capsulated. This leads us to the most important recent advance in the serology of the coliform group. Studies by Julianelle (76) on the immunological reactions of the unencapsulated cell supplied the hypothesis that the different organisms once deprived of the ability to elaborate capsular polysaccharide might be more readily amenable to systematization. Accordingly, unencapsulated, or "R" strains,[11] were derived from the encapsulated "S" strains by continued cultivation of the "S" form in homologous anti-S serum.

With such unencapsulated strains and such sera, Julianelle studied some of the encapsulated coliform bacteria (No type B Friedländer bacilli were included) and found that they fell into two main groups: one including Friedländer bacilli types A and C; and the other rhinoscleroma, ozaena, *A. aerogenes*, and granuloma strains. More recently (1938) Julianelle has tested three strains of *E. coli* and two of *A. aerogenes* in a preliminary study. The former fell into two groups and the latter into one. Such serological work is laborious and time-consuming, but it is possible that some such approach as this will prove very fruitful.

CLASSIFICATION OF THE COLIFORM BACTERIA

Malcolm (103) has stated that the coliform group of bacteria consists of a gradation of types so closely linked together as to render it undesirable to divide the group into two genera. The

[11] The designations of the culture phases are those of Julianelle (76).

reviewer hopes to convey a concept of coliform bacteria as a group of closely related, closely intergrading bacteria in which, by the dropping of one character or the acquisition of another, an organism appears as a new strain. It is only reasonable to suppose that at intervals along the gamut of numerous varieties one can pick out a strain that will differ in a number of respects from another selected from another locus in the series.

The error of past classifications has been to dignify each recognizable variety encountered with a name. We now know that some of our most cherished measuring rods, such as the methyl-red and Voges-Proskauer reactions, sugar fermentations, indole production, and the utilization of citrate as a sole carbon source, are not to be depended upon to give with the same organism at all times the same reaction. Coliform bacteria are particularly restless when compared with most other groups of bacteria. It is among them that the most interesting and numerous instances of variation, and "shifts" occur.

The most fundamental objection, from our point of view, to the establishment of more than one genus for the coliform bacteria is that to do so will obscure for all save a few who are unusually conversant with the group its essentially intergrading nature. The correct orientation and stimulus which this point of view provides should result in further needful research in the field.

It seems, furthermore, that the concept of the coliform "intermediates" is such that if we are to have more than one genus we must also recognize one for the "intermediates." To classify these forms with *Escherichia* is to obscure the significant points that characterize "intermediates" as such, and to lose sight of their essential intermediate nature.

For some time we have regarded *Klebsiella* as coliform strains derived from the more definitely recognized types of the group, such as *Aerobacter*, and differentiated from them by ecological factors to manifest a lessened and variable biochemical activity, a more distinct encapsulation and, in some instances at least, enhanced virulence. Serology indicates their close relationship to *Aerobacter*. *Aerobacter* is less a toxin producer than is *Escherichia*, and it is more easily degraded by environmental

influences. Such a form as Friedländer's bacillus is, according to Smith, a more advanced or developed pathogen than the toxin-producing *E. coli* which Smith speaks of as "a primitive aggressive form."

If the Friedländer bacillus has evolved beyond the primitive toxin stage, as conceived by Theobald Smith, it would require some sort of specialization in order to maintain its position successfully as an invader of respiratory membranes. This the capsule supplies. It would seem that there is little to be gained in setting up a number of species in this group based on very little else than host source.

Personally, we should like to follow Jordan (General Bacteriology, 11th edition, 1935) in retaining the genus *Bacterium* for the entire group of coliform bacteria. It seems, however, that there are taxonomic difficulties preventing this. For a genus name to be valid its type species must be recognizable, and *Bacterium triloculare* Ehrenberg, 1828, is of course unrecognizable. *Bacterium* was retained for some time as a temporary genus but it is now felt that the time has passed for a continuation of such a status (15). Were the taxonomists to propose one genus for all coliform bacteria, it is our suggestion that it should comprise the two species, now called *E. coli* and *E. freundii;* the two now designated as *A. aerogenes* and *A. cloacae;* and one of the six now listed under *Klebsiella*, presumably *K. pneumoniae.*

CONCLUSION

The coliform group of bacteria has presented distinct problems in classification in the past. As early as 1893 Denys and Martin (31) indicated the two main reasons for the encumberment of bacteriology with false species as, first, a paucity of comparative studies, and second, the variations to which a single species is subject. Sufficient data have now accumulated to warrant revision of former classifications. An understanding of the complexity of the group as revealed by the application of a wide variety of biochemical tests and an appreciation of its variability and changeability has convinced workers of the futility of attempting to give species rank to more than a few of the many

types that have been described. This point of view has been strengthened by the apparent failure thus far to demonstrate that certain strains are peculiar to a given environment or activity. It appears rather that coliform bacteria must be thought of as a well-nigh ubiquitous group of organisms. Of these, that

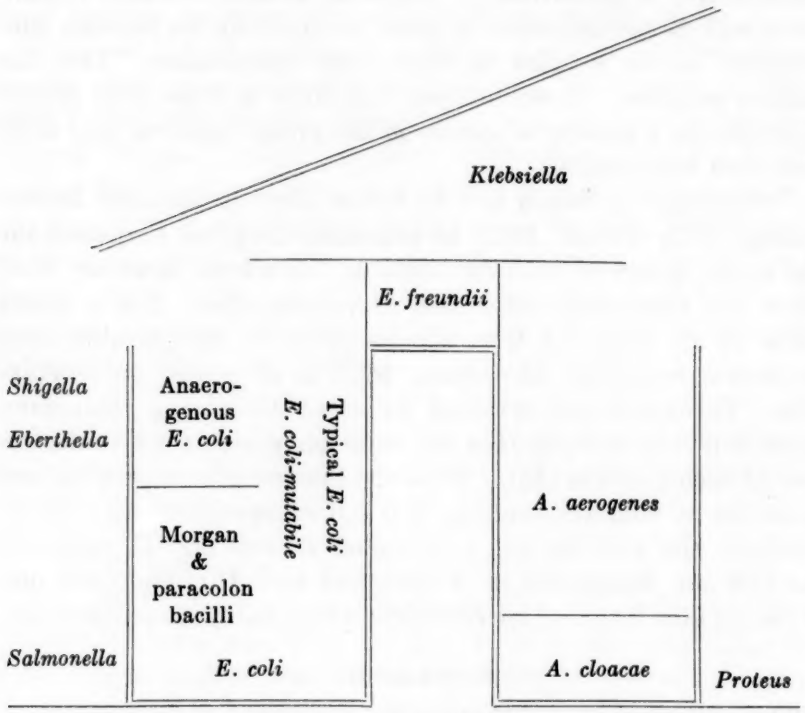

FIG. 1. A CONCEPT OF COLIFORM CLASSIFICATION

type will predominate in a given environment which is best adapted to the conditions of living found there.

The recent revision of Bergey's Manual of Determinative Bacteriology (12) has wisely taken cognizance of these principles and radically revised coliform classification in the direction of simplicity. The opportunity which this review offers for comment on that classification has been taken advantage of and suggestions have been made for further progress which our ac-

quaintanceship with the organisms and their literature would seem to warrant.

The coliform group is a large one made up of closely related, highly intergrading, and somewhat unstable bacteria which form a fairly wide gamut or *continuum* extending from the lactose-negative paracolon forms at one extreme to the highly reactive *A. aerogenes* at the other. Standing with the paracolon forms next to *Salmonella*, one finds the Morgan bacillus. In about the same position, and leading to *Eberthella* and *Shigella*, are located the anaerogenous *E. coli*. Next to these varieties come the slow lactose-fermenting *E. coli* so likely to be manifested as "unstable variants." Completing one side of the picture, one finds the typical *E. coli* which bridge over to the *A. aerogenes* side by way of the "intermediates." Below *A. aerogenes* we find *A. cloacae* which appears to point toward the genus *Proteus*, and above all forms, but particularly above *A. aerogenes*, are located the Friedländer organisms. We have tried to represent this concept graphically in figure 1.

It is felt that our present understanding of the coliform group requires for its best expression the allocation of all these bacteria to five species within one genus.

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QUANTITATIVE ABSOLUTE METHODS IN THE STUDY OF ANTIGEN-ANTIBODY REACTIONS

MICHAEL HEIDELBERGER

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City

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CONTENTS

The first comprehensive theories of antigen-antibody interaction were due to Ehrlich and to Bordet. The emphasis of Ehrlich's theory was placed on the chemical reaction of antigen with antibody, while that of Bordet's was laid on the adsorption of one by the other. Bordet's own modification of his theory and the formulation by Arrhenius and Madsen of the Ehrlich view in terms of reversible reactions which could be expressed on a mass law basis still left a wide gap between the theories. This gap was not bridged or accounted for by the commonly adopted use of colloid chemical terminology. In more recent years, however, adsorption has come to be regarded as a chemical, rather than a physical process, so that the old distinction between the Ehrlich and Bordet theories has become scarcely more than a point of view. The demonstration by Proctor and Wilson and especially by Jacques Loeb that the laws of classical chemistry could be applied to the behavior of typical colloids such as proteins has given direction and force to subsequent studies in innumerable fields of biological research, and in particular to

the studies on the mechanism of immune reactions on which most emphasis will be placed in this review.

At the time the Ehrlich and Bordet theories were formulated, and for many years afterward, little was known of the chemical nature of either antigens or antibodies. The fundamental researches of Landsteiner (1), the discovery of the immunologically specific polysaccharides by Avery and Heidelberger (2) and the ensuing work on artificial carbohydrate-protein antigens by Avery and Goebel (3), and many other studies which have been reviewed elsewhere (1, 4, 5, 6, 7) have led to a better understanding of the relation of chemical structure to antigenic function. As to antibodies, it is generally conceded that these are actually modified serum globulins and a considerable body of information as to their properties is already available (4–7). A modern theory of antigen-antibody interaction will therefore not only rest on a more secure understanding of the nature of the substances involved, but must also account satisfactorily, and in the last analysis quantitatively, for a very large accumulation of knowledge.

A cause of early interpretative difficulties in the study of antigen-antibody interactions, promptly recognized by Ehrlich and Morgenroth (8), was the common use of whole sera or other multiple component protein mixtures as "antigen." A few investigators, however, did not encounter these difficulties, and drew conclusions of far reaching importance. Owing doubtless to the preponderance of casein in milk the antisera obtained by Müller on injection of milk into rabbits (9) seem now to have behaved like antisera to a single antigen, and yielded a precipitin reaction zone in which neither antigen nor antibody could be detected in the supernatant. An eight- to ten-fold range of combining proportions was noted and evidence given of an actual soluble antigen-antibody compound in the non-precipitating region of a large excess of milk. Similarly, a four-fold zone of combining proportions in which neither component could be detected in the supernatant was observed by von Dungern (10) in studies on the precipitin reaction in rabbit antisera to octopus, crab, and mollusc plasmas. By the demonstration of hemo-

cyanin in the specific precipitate by the blue color and its reversible disappearance and reappearance with carbon dioxide and air, von Dungern was the first to show the value of the "marked antigens" which later were to figure so prominently in the study of the precipitin reaction. He also emphasized the presence of several distinct antibodies in the sera studied and commented on them in terms of the reactive groupings involved.

Although the broad range of combining proportions in antigen-antibody reactions had already been stressed by Danysz in explanation of the effect which he discovered in toxin-antitoxin interaction (11) and was later cited by Fleischmann and Michaelis in pointing out the fallacy of precipitin measurements by volume (12), these fundamental observations were neglected by Arrhenius and Madsen (13) in their comparison of antigen-antibody reactions with the union of weak acids and weak bases.

Until recently the only analytical methods available for the study of antigen-antibody interactions were either those based upon biological effects, with their large variation in individual animals, or the purely relative serological dilution methods, which, owing to their large capacities for subjective and other errors, have remained to this day essentially qualitative in spite of cumbersome precautions. Important steps toward the solution of analytical chemical difficulties in the study of the precipitin reaction were taken by Wu and his collaborators (14). The hemoglobin (Hb)-antibody and iodo-ovalbumin-antibody systems were studied. In the former, Hb, another "marked antigen," could be determined colorimetrically in the washed specific precipitate. Total nitrogen was also estimated by the Folin-Wu modification of the micro-Kjeldahl method, the two analyses affording a means of analyzing for both antigen and antibody in the precipitate. Although two analytical principles upon which later progress was made were thus laid down, the conclusions drawn from these obviously only tentative experiments were at variance with earlier and later well-founded observations and Wu unfortunately published nothing further along these lines.

QUANTITATIVE STUDY OF THE S III-ANTIBODY SYSTEM

In the meantime the writer had begun a study of the precipitin reaction and had found a modification of the Pregl micro-Kjeldahl nitrogen method both convenient and accurate. The study was then continued over a number of years, largely in collaboration with F. E. Kendall. The analytical difficulties were in part overcome by the use as "antigen" of the specific polysaccharide of Type III pneumococcus, the salt of a nitrogen-free polyaldobionic acid (15) which had been obtained in a state approaching analytical purity.[1] The analytical problem was further simplified by the use of partly purified antibody in the form of solutions prepared according to Felton (17) from Type III antipneumococcus horse serum. After "ageing" or stabilization, roughly one-half of the nitrogen in these solutions was found to be specifically precipitable by S III. Since the polysaccharide added in varying proportions contained no nitrogen, the difference between the original N content of the solution and that remaining after centrifugation of the precipitate gave an accurate measure of the precipitated antibody in absolute weight units instead of in the relative terms then customary (18). When it was found that identical results were obtained by direct analysis of the washed specific precipitate and that the amount of antibody-N precipitated was independent of the non-specific N or protein present (19–22) the more cumbersome measurements by difference were abandoned.

It was found that when a very small amount of S III was added to a relatively large amount of antibody, A, more than 240 mgm. of A might be precipitated for each milligram of S III. When increasing amounts of S were added to separate portions of A the ratio of S to A in the precipitate increased steadily, with no evidence of discontinuity. In this region of the reaction range no S could be found in the supernatant by the delicate serological test sensitive to S in dilutions of 1:10,000,000, so that it seemed

[1] Subsequently referred to as S III or S. All but the studies on rabbit sera described in a later section were carried out with polysaccharide preparations now known to have been degraded by heat (16). This influenced only the numerical values obtained, not the conclusions based on the data.

reasonable to assume that all of the S added was in the precipitate. In this region antibody was still in excess, as was shown by the addition of a little S to the supernatant. When still larger quantities of S were added to the same amount of A a region of the reaction range followed in which neither S nor A, or only a minimal amount of each, was demonstrable in the supernatant from the precipitate. We have termed this region, often of considerable extent, the "equivalence zone." With still larger amounts of S, the latter finally appeared in the supernatant, and in this region precipitation of antibody remained at a maximum while more and more of the S added entered into combination until in some sera constant composition was attained. Finally, excessive amounts of S III caused the formation of less and less precipitate in an "inhibition zone," until precipitation was entirely prevented. The reaction course, except for the inhibition zone, is illustrated by the curves in figure 1.[2] It had previously been found (21) that such reaction curves, up to the equivalence zone, could be expressed by the empirical equation

$$\text{mgm. antibody N precipitated} = a\,S - b\,S^2 \quad \ldots\ldots\ldots [1]$$

These reactions were found to be reversible in the sense that the precipitate formed in the region of excess antibody took up S when shaken with a solution of the polysaccharide and even dissolved in concentrated S solutions. The reversible shift in composition of the hemocyanin precipitate in either direction with antigen or antibody had been shown long before by von Dungern (10). It therefore seemed reasonable to postulate the following equilibria in the four limiting regions of the reaction range: at extreme antibody excess, $S + 4A \rightleftharpoons \underline{SA_4}$; at the midpoint of the equivalence zone, $S + A \rightleftharpoons \underline{SA}$; in the antigen excess region, $\underline{SA} + (x - 2)S \rightleftharpoons \underline{S_{x-1}A}$; and in the inhibition zone, $\underline{S_{x-1}A} + S \rightleftharpoons S_xA$. The underlined formulas represent precipitates, and in all formulas the composition is expressed in arbi-

[2] This figure was also used in an article on "Chemical Aspects of the Precipitin and Agglutinin Reactions" read before the American Chemical Society's Symposium on the Physical Chemistry of Proteins at the Milwaukee Meeting, Sept., 1938; Chemical Reviews, 1939, **24**, 323.

trary units, not molecules. In the first two equations equilibrium must lie far to the right as measurable dissociation could not be detected. It was shown that S_xA contained one more molecule of S than the precipitate with which it was in apparent equilibrium (18), confirming the belief of Müller (9), von Dungern (10), and Arrhenius (13) in a soluble antigen-antibody compound

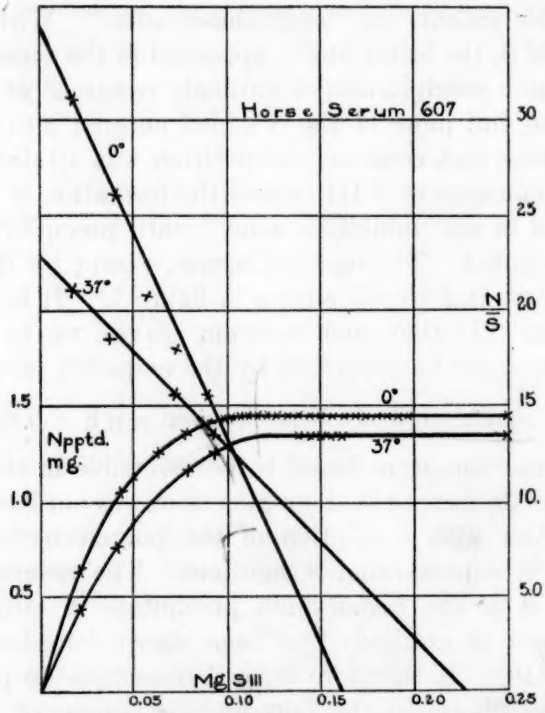


FIG. 1.[2] Curves: Antibody N precipitated from antipneumococcus Type III horse serum by increasing amounts of S III. Lines: Ratios of antibody N:S III in precipitates, over range indicated by crosses.

in this zone, rather than the obscure "peptization" of the precipitate which had been proposed by advocates of the colloidal theory despite Müller's direct chemical evidence to the contrary.

Since S III is the highly ionized salt of a polymeric aldobionic acid (15), and antibody globulin, dissolved in physiological media, probably exists as an ionized sodium chloride complex, the initial reactions, at least, may be ionic. The application of

the mass law in some form would seem justified. The precipitin reaction between S III and homologous antibodies would then be merely a complex instance of a specific precipitation such as that between barium and sulfate ions or silver and cyanide ions. Even the inhibition zone would have at least a partial analogy in the well-known solubility of silver cyanide in excess cyanide solution.

In qualitative terms this interpretation of the precipitin reaction appears satisfactory, but difficulties arise in the quantitative formulation of the reaction in terms of the law of mass action. It might, for example, be expected that there would be definite steps or stages between the limit of SA_4 in the region of large antibody excess and SA in the equivalence zone, also in the inhibition zone, but such abrupt changes in composition are not found. This might be explained by assuming a continuous series of solid solutions, or that the mutual multivalence of S and A is so great as to permit formation of a continuous series of compounds. There are, however, valid objections to these views in spite of the well-founded structural (15) and other evidence (24) that S III contains a number of recurrent immunologically reactive groupings or valences, and the present-day views as to the structure of proteins (25), which are in entire accord with the assumption of recurrent groups of amino acids which might be "valences" or the centers of specific combination. The principal difficulty in the formulation of the reaction along these lines lies in the finding that the composition of the precipitate depends upon the proportions in which the components are mixed, and not upon the antibody concentration at equilibrium, or at the end of the reaction (23). This remarkable state of affairs, illustrated in table 1, does more than prevent a simple treatment of the precipitin reaction according to the law of mass action, for it also prevents characterization of this and other immune reactions by adsorption isotherms, as has been attempted from time to time, for adsorption isotherms also contain a concentration term.

Another difficulty in the quantitative formulation of the reaction was due to the realization that the anticarbohydrate in Type III antipneumococcus horse sera was not a single substance,

but a mixture of antibodies of greatly differing reactivities. This was clearly shown (23) by the presence of residual, difficultly precipitable antibody after the serial addition of small quantities of S III, the occurrence of a portion of antibody precipitable at 0° but not at 37°, and the precipitability of only a part of the antibody by S III which had been methylated.

TABLE 1*

Effect of volume and final concentration of antibody on antibody N precipitated

VOLUME	ANTIBODY B 62 AT 0°C.		ANTIBODY B 61 AT 37°C.	
	Antibody N pptd. by 0.03 mgm. S III	Final concentration antibody N	Antibody N pptd. by 0.05 mgm. S III	Final concentration antibody N
ml.	*mgm.*	*mgm. per ml.*	*mgm.*	*mgm. per ml.*
2	0.87	0.21		
4	0.91	0.10	0.87	0.25
6	0.87	0.07		
8	0.84	0.06	0.87	0.12
10	0.84	0.05		
12	0.87	0.04	0.85	0.08

* Adapted from J. Exp. Med., 1935, **61**, 563.

QUANTITATIVE FORMULATION OF THE S III-ANTIBODY SYSTEM

With the aid of several assumptions, however, it was found possible to derive from the law of mass action a relation which accounts quantitatively for the S III-antibody reaction (23) and many other instances of the precipitin reaction as well. These assumptions and simplifications are:

1. S III and antibody (A) are chemically and immunologically multivalent with respect to each other; that is, each substance possesses two or more groupings capable of reacting with the other.

2. Although the anticarbohydrate is known to be a mixture of antibodies of different reactivities it may be treated mathematically as if its average behavior were that of a single substance, A.

3. For convenience of calculation the S III-antibody reaction is considered as a series of successive bimolecular reactions which take place before precipitation occurs.

4. The mass law applies, so that the rates of formation of the reaction products are proportional to the concentrations of the reacting substances.

The reactions postulated are, in arbitrary units:

$$S + A \rightleftharpoons SA \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots [2]$$

followed, for example in the region of excess antibody, by the competing bimolecular reactions due to the mutual multivalence of the components:

$$\begin{aligned} SA + A &\rightleftharpoons SA \cdot A \\ SA + SA &\rightleftharpoons SA \cdot SA \end{aligned} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots [3]$$

A third step would follow, in which the competing bimolecular reactions would be:

$$\begin{aligned} SA \cdot A &+ A &&\rightleftharpoons SA \cdot A \cdot A \\ SA \cdot SA &+ A &&\rightleftharpoons SA \cdot SA \cdot A, \\ SA \cdot A &+ SA \cdot A &&\rightleftharpoons SAA \cdot SAA, \\ SA \cdot A &+ SA \cdot SA &&\rightleftharpoons SAA \cdot SA \cdot SA, \text{ and} \\ SA \cdot SA &+ SA \cdot SA &&\rightleftharpoons SASA \cdot SASA \end{aligned} \quad \ldots\ldots\ldots\ldots [4]$$

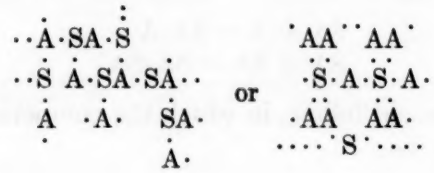
in which the first two reactions would occur only in the presence of enough A to carry the composition of the reaction product beyond the SA_2 stage. Similarly, each compound formed in the third step would react with each other compound, or with more A, if present, to form still more complex substances, and the reaction would continue until particles would be formed large enough to settle from the solution. Precipitation would take place at this point, doubtless facilitated by the mutual discharge, with loss of affinity for water, of ionized or polar groupings brought together by the series of chemical reactions (cf. also 4).

If A and S III are mixed in equivalent proportions the SA formed in reaction [2] would merely polymerize in steps [3], [4] . . . , and the equivalence-point precipitate would be $(SA)_n$.

In the region of excess S III similar expressions would apply, in which S and A would be interchanged in [3], [4], ... In the presence of a large excess of S, in the inhibition zone, there would also be present in solution a soluble compound, S_xA, containing

one more molecule of S in combination than the last insoluble compound (18). Since this is formed only with a very large excess of S, all of the specific groupings of A would tend to react with S rather than with SA complexes and there would be no large, insoluble intermolecular aggregates formed.

The final precipitate, then, would in each case consist of antibody molecules held together in three dimensions by S III molecules,



a view similar to that put forward also by Marrack (4) but, it is believed, more definite and more easily treated quantitatively. The process of aggregation as well as the initial hapten-antibody combination is considered to be a chemical reaction between definite molecular groupings.

Since no evidence of dissociation could be found over a large part of the reaction range, the equilibria postulated evidently lie very far to the right. The mathematical treatment of these reactions has been given elsewhere (23). In applying the derived equations to experimental data it is necessary to convert units of S and A into milligrams. This is accomplished by defining one unit of antibody N as 1 milligram. A may then be put equal to the number of milligrams of antibody N precipitated at the equivalence point (midpoint of the equivalence zone) and R equal to the ratio of A to milligrams of S III precipitated (added) at the same reference point. The equation most frequently applicable then becomes

$$\text{mgm. of antibody N precipitated} = 2RS - \frac{R^2S^2}{A} \quad \ldots\ldots [5]$$

The theoretical significance of a and b in the empirical equation [1] is now clear, for $a = 2R$ and $b = \frac{R^2}{A}$. Both of these constants have the immunological and chemical significance given above.

If, instead of the difficultly determinable "equivalence point" the reference point for R and A be taken as either end of the equivalence zone, depending on the precipitin system studied, it will usually be found possible to avoid the complicated R − 3R equations also given in (23) and use only the simpler R − 2R equation [5]. Table IX in (23) shows the agreement between found and calculated values to be very close for a number of antibody solutions and sera. In the use of equation [5] it is assumed that all of the antibody is precipitated at the reference point. If this is at the beginning of the equivalence zone, as in the S III - A system, the assumption is not entirely correct, for the amount of A precipitated does not reach a maximum experimentally until S is present in appreciable excess. For the complete description of the behavior of a serum in the precipitin reaction a separate determination of the maximum amount of specifically precipitable nitrogen is necessary.

If both sides of equation [5] be divided by S, the resulting equation,

$$\frac{\text{mgm. antibody N precipitated}}{\text{mgm. S precipitated}} = 2R - \frac{R^2}{A}S \quad \ldots\ldots\ldots [6]$$

is that of a straight line. This linear relationship makes it possible to characterize an unknown Type III antipneumococcus serum or antibody solution in the region of excess antibody by two or, better, three analyses, in duplicate. If the ratio of antibody N to S III precipitated be determined for two or three different amounts of S III in the region of excess antibody and a straight line be drawn through the points so obtained, the intercept on the y axis = 2R and the slope = $-\frac{R^2}{A}$. With the R and A values at the beginning of the equivalence zone calculated in this way the amount of antibody nitrogen precipitated by any quantity of S III less than $\frac{A}{R}$ may be calculated with a fair degree of accuracy. (For the linear relation, also, see Fig. 1.)

In the region of excess S III the behavior of a serum as far as the beginning of the inhibition zone may be characterized by the determination of the A and S III precipitated at two points,

since in this region the terms of equation [6] may be inverted and the linear relation

$$\frac{\text{S pptd.}}{\text{A}} = 2\text{R}' - \frac{(\text{R}')^2\text{A}}{\text{Total S}}$$

applies if R′ be taken as the $\frac{\text{S}}{\text{A}}$ ratio at the end of the equivalence zone at which S III appears in excess,[3] A be taken as the amount of antibody N precipitated, and $\frac{\text{S pptd.}}{\text{A}}$ be plotted against $\frac{1}{\text{Total S}}$.

In the inhibition zone, in which large quantities of S III are present and the amount of precipitate has begun to diminish, this equation is no longer applicable and it is necessary to determine the apparent dissociation constant of the soluble compound S_xA (cf. also (26)).

Despite the wide variation in the behavior of individual sera the above expressions permit the complete description of the precipitin reaction between S III and an unknown antiserum without an unduly burdensome number of microanalyses or the sacrifice of a large amount of material.

In all of the experiments described above horse sera or antibody solutions obtained from horse sera were used, but equations of the same form, with constants of smaller magnitude, were found to hold as well for antipneumococcus Type III sera produced in rabbits (27).

PRECIPITATION IN PROTEIN-ANTIPROTEIN SYSTEMS

Extension of these studies to protein-antiprotein systems was more complicated, since it was necessary to distinguish between antigen nitrogen and antibody nitrogen if the composition of the

[3] In (23) p. 590, "total S" in this equation was taken as the (constant) amount of S combined with A precipitated at this reference point, while A in the equation was made to vary by defining it as that portion of the constant amount of antibody precipitated throughout this zone given by the fraction $\frac{\text{"total S"}}{\text{S added}}$. Owing to space limitations this explanation was unfortunately omitted.

precipitate was to be directly determined. This was accomplished by the use of a red protein dye, R-salt-azo-biphenyl-azo-crystalline egg albumin, which was freed from fractions reactive in most anti-egg albumin sera and then injected into rabbits (26, 28, 29). In the specific precipitates produced by the dye and antibody, antigen was estimated colorimetrically after solution of the washed precipitate in alkali. The entire solution was then rinsed quantitatively into a micro-Kjeldahl flask for a total nitrogen determination, after which the amount of antibody nitrogen could be calculated by deducting from the total N the amount of dye-antigen N found colorimetrically. Equations [5] and [6] were applicable in this system as well, also an empirical equation,

$$\text{mgm. antibody N pptd.} = 3RD - 2\sqrt{\frac{R^3D^3}{A}} \quad \ldots\ldots\ldots [7]$$

in which R = the A:D ratio at the maximum for antibody N precipitated, D = the amount of dye N precipitated, and A = the maximum precipitable antibody N. This equation permitted calculation of the maximum specifically precipitable nitrogen with avoidance of a separate set of analyses for the determination of this constant. In this system the composition of the precipitate could be estimated by direct analysis over the entire reaction range, and the ratios of the components were found to vary without discontinuity from higher to lower A: dye values as relatively larger amounts of dye were added to the antisera. This was also shown by the increasing redness of the precipitates. Azoprotein-antibody systems were also studied by Marrack and Smith (20b) and by Haurowitz and Breinl (30). Both groups confirmed the varying composition of the precipitate, the latter workers having previously reached similar conclusions regarding hemoglobin-antibody precipitates (31).

With the aid of the information gained from the precipitin reaction between R-salt-azo-biphenyl-azo-crystalline egg albumin and its homologous antibody it was found possible to study a colorless protein, crystalline egg albumin, and its homologous antibodies. This instance of the precipitin reaction was also

found to be quantitatively described over a large part of the reaction range by the theory, and the equations were applied to unorganized analytical data accumulated by other workers (32). The crystalline horse serum albumin (33) and mammalian thyroglobulin systems also behaved in accordance with the theory (34). The precipitin reactions of antipneumococcus sera other than Type III were likewise found to be described by the same equations derived from the theory (27, 35), and these relations were also found by Pennell and Huddleson (36) to cover the reactions of anti-*Brucella* goat sera with the appropriate antigens.

With hog thyroglobulin and antisera produced in rabbits (34) it was possible to test directly an assumption made with S III, crystalline egg albumin, and crystalline serum albumin. It had been postulated that in the region of excess antibody and in the equivalence zone all of the presumably pure hapten or antigen added was precipitated if the exceedingly sensitive precipitin test on addition of more antibody to a portion of the supernatant failed to reveal the presence of antigen. This assumption had been criticized by Taylor, Adair, and Adair in the course of their studies on the egg albumin system (37). A direct test with egg albumin did not seem easy to devise, but with highly purified thyroglobulin (38) it was found that 96 to 101 per cent of the iodine added was precipitated in the region of excess antibody. These observations and the quantitative data recorded (34) also render untenable the view subsequently advanced by Clutton, Harington, and Yuill (39) that thyroglobulin is not an antigen.

The quantitative precipitin technique affords an exceedingly accurate method for the estimation of minute quantities of specific polysaccharides (21) and of small quantities of proteins (32). Determination of the amount of specific nitrogen precipitated in the region of antibody excess from a previously calibrated antiserum permits the quantity of antigen in the sample to be read off from the specific nitrogen (antigen N + antibody N) calibration curve. The amount of nitrogen actually measured is usually many times that due to the antigen, so that very small quantities of antigen may be accurately determined. This method has been applied to the estimation of albumin and globu-

lin in body fluids by Goettsch and Kendall (39A), and has been used as a guide for the isolation and identification of protein fractions from normal and pathological human sera by Kendall (39B).

SPECIFIC BACTERIAL AGGLUTINATION

At the inception of the present work there was available for the study of the mechanism of bacterial agglutination no quantitative method conforming to the criteria of analytical chemistry. It was found that the microanalytical technique used in the case of the precipitin reaction could be modified so as to permit the estimation of agglutinins for pneumococcus with a high degree of accuracy. A measured amount of thoroughly washed, killed pneumococcus M (S) (40–2) or S (R) (43) suspension was analyzed for nitrogen. This value was deducted from the nitrogen found after the same volume of cell suspension had been agglutinated by an accurately measured volume of serum and washed free from non-specific protein. The difference gave, in milligrams per milliliter, the amount of agglutinin nitrogen removed by the bacterial cells under the conditions used, and, when the proportions of pneumococci and serum were chosen so as to leave the cells in excess, gave the agglutinin content of the serum in absolute, not relative, terms. As in the precipitin reaction, the amount of agglutinin found was shown to be independent of the non-specific protein present, and to depend on the relative proportions in which the components were mixed and not on the final concentration of antibody. Though Type I pneumococcus and homologous antibody reacted according to the same type of equation as in the precipitin reaction, the agglutinin reaction was actually found to be simpler, since the exigencies imposed by the reactive bacterial surfaces limited the range of combining proportions of bacterial polysaccharide and antibody (42). As might have been anticipated from the view that specific bacterial agglutination differs from specific precipitation only in that the former reaction takes place on particulate matter, the latter between two dissolved reagents (44), type-specific pneumococcal anticarbohydrate was found to be quantitatively the same

whether measured as agglutinin or as precipitin. It was also found that the entire course of these instances of specific bacterial agglutination could be accounted for, as in the precipitin reaction, on the basis of a chemical reaction between multivalent antibody and multivalent antigen, without assumptions as to electrical potential or cohesive force such as those made by Northrop and de Kruif (45).

The quantitative methods introduced were found applicable in several instances of cross reactions as well, and yielded data showing the wide variations in the reaction course when compared with the corresponding homologous reactions (29, 36). The cross reactions between Types III and VIII pneumococci, their type-specific polysaccharides, and Types VIII and III antipneumococcus sera were shown to involve a relatively small proportion of the total antibody, especially in sera produced in the rabbit (46). This was taken to indicate that the immunological (chemical) unit responsible for the specificity of each of the two polysaccharides involved was larger than the simple glucuronic acid or aldobionic acid portion common to both, as had been maintained up to that time (47), and this view has now been accepted (48).

SOME CONSEQUENCES OF THE QUANTITATIVE THEORY

The quantitative theory of the precipitin and agglutinin reactions discussed above was proposed by its authors, in avowed realization of many of its weaknesses, as a makeshift and a first attempt at a general quantitative theory of two important immune reactions. On this basis it has not only explained much that is not accounted for by the older, essentially qualitative theories, but has also served as a working hypothesis that permitted several rather far-reaching predictions which might not otherwise have been foreseen.

In the first place the method for the estimation of the maximum specifically precipitable nitrogen, the absolute measure of the precipitin content of a serum, was the outgrowth of the initial oversimplification in which direct application of the mass law was attempted (18). The earlier oversimplification did not survive (cf. 20, 23), but the method based upon it is a standard one to-day.

In the second place, a study of the effect of strong salts on the reaction between pneumococcal polysaccharide and homologous antibodies (49) showed that the lessened precipitation and decrease (table 2) in the values of both constants in Equation [5] was not due to increased solubility of the precipitate. On the basis of the quantitative theory (23) a reversible shift in the equilibrium between polysaccharide and antibody was indicated,

TABLE 2

Effect of the concentration of sodium chloride upon the reaction between S III and antibody

FINAL NaCl CONCENTRATION	HORSE ANTIBODY SOLUTION B 36					RABBIT ANTIBODY SOLUTION B 50*	
	0.1 M	0.15 M	0.51 M	0.93 M	1.79 M	0.15 M	0.93–0.98 M
S III used	Nitrogen precipitated						
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0.02	0.54	0.50	0.42	0.39	0.36		
0.05	1.13	1.03	0.90	0.84	0.75	0.43	0.24
0.075	1.41	1.41	1.29	1.15	1.03	0.60	
0.10	1.75	1.66	1.54	1.28	1.22	0.77	0.34
0.15	1.78	1.86	1.62	1.50	1.45	1.04	0.39
0.20†	1.82	1.85	1.70	1.58	1.51	1.18	0.41
Equations; mgm. antibody N pptd...	$27.5\,S - 104\,S^2$	$25\,S - 84\,S^2$	$22.2\,S - 72\,S^2$	$20.2\,S - 68\,S^2$	$18.1\,S - 57\,S^2$	$9.5\,S - 18\,S^2$	$5.0\,S - 15\,S^2$
A‡.....	1.82	1.86	1.71	1.50	1.44	1.25	0.42

* Prepared according to Felton, J. Immunol., 1931, **21**, 357.

† Excess S III.

‡ Calculated mgm. antibody N pptd. at antibody-excess end of equivalence zone. (Reprinted from J. Exp. Med., 1936, **63**, 819.)

and it was predicted that this shift would permit the isolation of pure antibody. For example, 0.1 mgm. of S III precipitated 1.24 mgm. of antibody N from a given serum in physiological saline (0.15 *M* NaCl), but formed an insoluble compound with only 1.01 mgm. of antibody N in 1.75 *M* NaCl. On the assumption that the equilibria involved were reversible, it was considered possible that if the reaction were first carried out in 0.15 *M*

NaCl, the non-specific protein were then washed out, and 1.75 *M* NaCl were next added, 0.23 mgm. of N should be dissociated in the form of pure antibody (49). Actually, antibody solutions of 90 to 98 per cent purity

$$\frac{\text{specific precipitin N} + \text{agglutinin N}}{\text{Total N}}$$

were readily attainable in this way in a single step from many unrefined antipneumococcus horse and rabbit sera of various types (50). With improvements in technique analytically pure antibody globulin was isolated (51, 52) through the use of the method. Studies on the purified antibodies led to the discovery that pneumococcal anticarbohydrate produced in the horse had a high molecular weight, while the same antibody (also anti-egg albumin) produced in the rabbit was of the size of the principal component of normal globulin. This phase of the work, which has been reviewed elsewhere (7), and accompanying quantitative studies (27) furnished much of the theoretical background (cf. also (53)) and practical methods of control for the use of anti-pneumococcus rabbit sera (53) and antibodies (54) in the treatment of pneumonia.

Verification of another prediction, made from the quantitative agglutinin theory, puts the function of salts in this immune reaction on a basis different from the currently held view. The reversibility of the precipitin reaction, in the sense that a precipitate may be shifted from one region of the reaction range to another by addition of antigen or antibody, warranted the assumption of a similar reversibility for the closely related agglutinin reaction. For example, Type I pneumococci may be agglutinated with a large excess of antibody, and the excess of antibody then removed by thorough washing and the agglutinated pneumococci resuspended evenly in saline. According to the theory, the prediction may be made that addition of an appropriate amount of Type I pneumococci or of Type I specific polysaccharide will cause reagglutination into larger clumps. This would be brought about through the chemical linkage of multivalent antibody on the agglutinated, washed cells either with multivalent S I on the

freshly added pneumococci, or with dissolved S I if a solution of the polysaccharide were added instead. It may also be predicted that if Type II pneumococci or Type II polysaccharide be added to a similar suspension of agglutinated, washed, Type I cells, reagglutination into larger clumps will not occur, although salt concentration, electrical potential, and cohesive force (45) would be identical, or nearly so, in the two sets of experiments. It may also be predicted that addition of Type I pneumococci *after* the Type II cells added in the preceding instance will result in reagglutination, leaving a turbid supernatant containing most of the Type II cells. All of these predictions are fully verified when subjected to experimental test (42), and the verification is interpreted in the light of the above theory as follows:

Specific bacterial agglutination is not a mere combination or coating of bacterial surface antigen with dissolved antibody, followed by non-specific flocculation due to the presence of salts, but appears more reasonably to be a more dynamic process: the chemical combination of multivalent antigen on the reactive bacterial surfaces with multivalent antibody, originally in solution, to build up larger and larger aggregates until these flock out and the process is terminated. The function of salts in this process is then the purely secondary one of minimizing electrostatic effects due to the presence of many ionized groupings on the particles, effects which might interfere with the primary process of building up aggregates by chemical interaction. At least in the case of the water-insoluble antibody to pneumococcus produced in the horse, salts also provide ions for the soluble, ionized salt complexes in which form this antibody probably reacts.

Even though the initial bimolecular antigen-antibody reaction on the bacterial surface may take place in the absence of electrolyte, the reactants carry ionized groups and it is evident that the succeeding competing bimolecular interactions between polysaccharide molecules on partly sensitized cells and additional antibody in solution or on other cells would soon result in the formation of particles carrying large numbers of ionized groups. Coulomb forces on such particles, in the absence of electrolyte,

are known to cause abnormally great viscosities and Donnan effects, so that it would not be surprising if these forces would prevent the continuation of the chemical reactions resulting in the completion of what is commonly recognized as specific bacterial agglutination. Only when the effect of these forces is reduced by a sufficient ionic atmosphere, on addition of electrolyte, is it possible to obtain significant figures for viscosity, osmotic pressure, sedimentation constants, and the like. To ascribe a similar rôle to electrolytes in specific bacterial agglutination would seem reasonable and consistent, for after reduction of the Coulomb forces the growing particles could again interact chemically, and the process of agglutination be completed. An analogous explanation of the function of salts in the reaction between heterogenetic antigen and antibody had already been given by Brunius (55) but this was not known until after publication of (42).

It will be noted that the effect ascribed to salts is essentially the same as in the older hypothesis. However, recognition of this effect as dependent upon the building up of aggregates between multivalent antigen and multivalent antibody simplifies the problem, abolishes the uncertainties and inconsistencies of the older view, and permits the precise definition of the conditions for specific bacterial agglutination. Whether or not a given antigen-antibody mixture will agglutinate, and which components, if any, will not be carried down when the (specific) aggregation takes place may now be predicted on the basis of the theory. Moreover, the prediction may be made even though the potential and cohesive forces in agglutinating and non-agglutinating systems are essentially similar.

In the above reagglutination experiments it may be considered that in the initial agglutination of relatively few pneumococci with relatively much antibody the dynamic process of combination of multivalent bacterial antigen with multivalent antibody has been interrupted at an early stage. In accord with this are the small size of the clumps formed and the ease with which the agglutinated cells may be resuspended, just as in precipitin reactions carried out with an excess of antibody the specific

precipitate may be relatively easily homogenized and resuspended. In the experiments under discussion the dynamic agglutination process was then continued under controlled conditions. It was found that resumption of agglutination of the Type I pneumococci could occur only when the chemical reaction of multivalent S I with multivalent antibody could go to completion, and that introduction of a chemically unrelated antigen such as Type II pneumococci or S II produced no effect, even though the potential had been suitably lowered by the presence of salt. These experiments are in entire agreement with the

TABLE 3*

Molecular composition of specific precipitates from rabbit antisera

ANTIGEN	EMPIRICAL COMPOSITION OF SPECIFIC PRECIPITATE				COMPOSITION OF SOLUBLE COMPDS. IN INHIBITION ZONE
	At extreme antibody excess	At antibody excess end of equivalence zone	At antigen excess end of equivalence zone	In inhibition zone	
Ea	EaA_5	EaA_3	Ea_3A_5	$\rightarrow EaA_2\rightarrow$	(EaA)
DEa	$(DEaA_5)$	$(DEaA_3)$	DEa_2A_5	$\rightarrow DEa_4A_3$	DEa_2A ?
Sa	SaA_6	SaA_4	SaA_3	$\rightarrow SaA_2\rightarrow$	(SaA)
Tg	TgA_{40}	TgA_{14}	TgA_{10}	$\rightarrow TgA_2\rightarrow$	(TgA)
S III	SA	S_3A_2	S_2A	$\rightarrow S_4A$	S_3A

Ea = cryst. egg albumin (32); DEa = dye egg albumin (26); Sa = cryst, serum albumin (33); Tg = thyroglobulin (34); S III = pneumococcus, Type III. specific polysaccharide (27).

A = Antibody, S = Minimum polysaccharide chain weight reacting. Data in parentheses are somewhat uncertain.

* Reprinted from the Journal of the American Chemical Society, 1938, **60**, 242.

conception of specific bacterial agglutination given above and also support Topley, Wilson, and Duncan's experiments (56) leading to the same conclusions.

If these conclusions are valid it is possible that the so often cited analogies between specific immune aggregation and the aggregation of suspensions in general have been misleading in their emphasis on a supposedly non-specific phase in the process. It is possible, also, that the knowledge gained in the quantitative chemical study of these immune reactions will be of service in clarifying the behavior of other systems of colloidal suspensions,

in which the chemical reactions involved in aggregation are far less well defined and understood. If the tables were turned in this way it would not be without its elements of humor.

Another outcome of these quantitative and theoretical studies has been the possibility of calculating, for the first time, the actual molecular composition of the specific precipitate at the principal reference points and in the principal zones of the entire precipitin reaction range. Marrack and Smith had calculated that at the flocculation optimum one molecule of pseudoglobulin antigen combined with about four molecules of antibody (20a), but use of more recent data on the molecular weights of antibodies (58, 59) has permitted the assignment of empirical formulas (table 3) over much of the reaction range in a number of systems (60). While these formulas cannot be considered as those of definite chemical compounds conforming to all criteria of homogeneity they represent faithfully at least the empirical composition of the specific precipitate at the reference points chosen and, in general, lie within such limits as to justify the application of classical chemical treatment to the study of the precipitin reaction.

MOLECULAR FORMULAS FOR ANTIGEN-ANTIBODY COMPOUNDS

With the use of the formulas in table 3 as a basis, a two-dimensional and therefore necessarily incomplete graphic representation of the entire reaction range between crystalline egg albumin (Ea) and antibody (A) might be offered (fig. 2). In this scheme the possibility of the combination of Ea with six molecules of A is taken to indicate that ordinarily up to six molecular groupings, not necessarily all different, in the Ea molecule may react with A; in other words that Ea has six immunological (chemical) valences, or a multiple of six. For simplicity the assumption is also made that the average A molecule has two valences or combining sites for Ea, but the possibility of additional bonds is not excluded.

The egg albumin studies (32, 60) have also shown that the immunological "valence" of the Ea depends to some extent on the breadth of reactivity of the antibody, and that this, in turn, generally varies with the length of the immunization period to

which the animal furnishing the antibody is subjected. If the minimum immunological "valence," or number of combining groups, of the antibody entering into specific precipitation is 2, it is probable that this increases during the course of immunization as the antibody becomes capable of reacting with more and more groupings on the antigen molecule. This is merely the expression, in chemical terms, of the well-known overlapping of specificities on prolonged immunization. Many antisera also contain antibody which behaves as if it possessed only a single immunologically reactive grouping, since it does not precipitate

Compounds in the Region of Excess Antibody

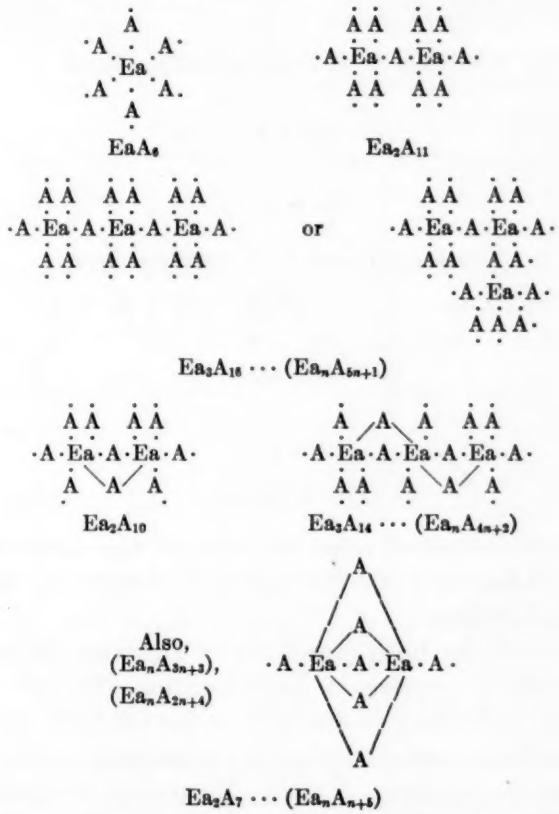
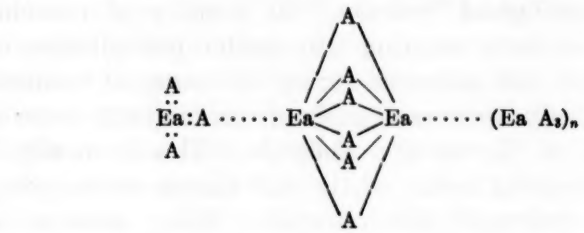
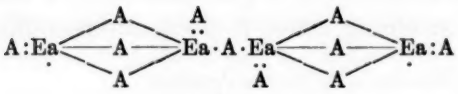


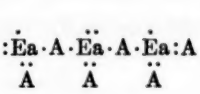
FIG. 2. MOLECULAR FORMULAS OF EGG ALBUMIN-ANTIBODY COMPOUNDS INDICATED BY ANALYSES AND THE QUANTITATIVE THEORY

Compounds in the Equivalence Zone



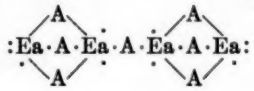


$Ea_4 A_{11}$



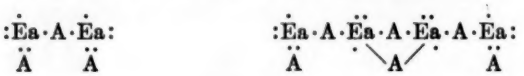
$(Ea\ A_2)_n$

Compounds in the Region of Antigen Excess



$Ea_4 A_7$, etc.

Soluble Compounds in the Inhibition Zone



$(Ea_2 A_3)_n$,

also, possibly

$(Ea\ A)_n$

FIG. 2, *concluded*

antigen when separated from the rest of the antibody, but is capable of adding to a specific aggregate formed by multivalent antibody and antigen.

The tendency for both constants of equation [5] to increase during successive courses of immunization (32, 27) reflects a change in the combining proportions of the antibody in the region of excess antibody, and a change in the opposite direction in the region of excess antigen. This is illustrated in figure 3, from which it will be noted that in the region of excess antibody more

and more antibody in the antisera from successive courses of injections is required to precipitate a given amount of Ea. Pre-

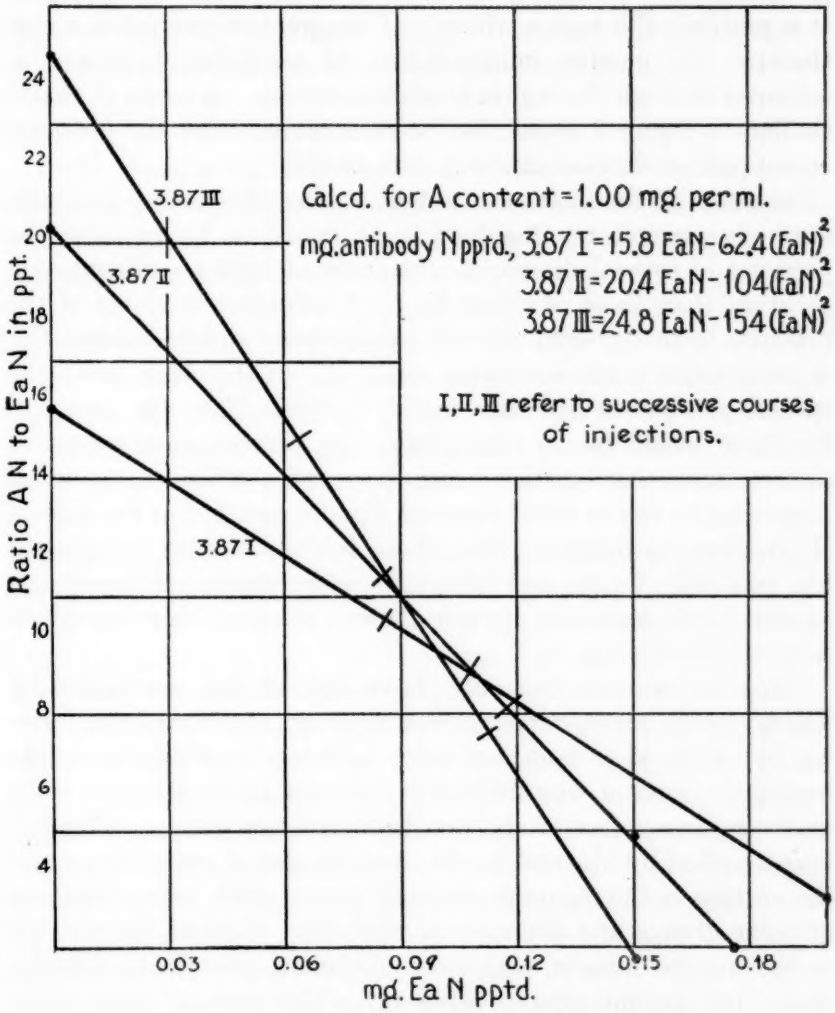


FIG. 3. Adapted from J. Exp. Med., 1935, **62**, 697

sumably for the same reason, that is, the probable ability of the antibody to react with more and more groupings in the Ea molecule as immunization proceeds, this tendency is reversed in the

region of excess antigen. In this zone the antibody becomes more and more efficient with continued immunization in that a given amount of antibody combines with more and more antigen. It is precisely this region which is of the greatest interest in serum therapy, for passive immunization of an infected patient is achieved through the region of antigen excess. A series of charts similar to figure 3 could also be constructed from the pneumococcal polysaccharide-antibody data in (27).

Extension of the molecular weight studies (58) to egg albumin-antibody precipitates dissolved in an excess of Ea revealed the presence of several dissolved substances of higher sedimentation constant than those of either Ea or A, affording evidence of the presence in this system, as well, of relatively simple, soluble Ea-A compounds in the inhibition zone. If, on the other hand, the specific precipitate had been merely "peptized" by the excess of Ea there would be no reason why the process should stop at discrete molecular entities larger than either of the components. According to recent calculations of Tiselius and Kabat made from electrophoresis diagrams (61) these inhibition zone compounds are probably Ea_2A_3 and $(Ea_2A_3)_2$, with higher polymers also present if the solutions are not allowed to stand long enough to come to equilibrium.

These experiments not only throw light on the mechanism by which specific precipitates are dissolved or prevented from forming by excess of antigen, but even furnish a possible clue to the vexing question of why inhibition does not likewise occur at the antibody excess end of the precipitin reaction range. This has been ascribed by Marrack to the close-packing of antibody around the antigen in the region of antibody excess, with consequent loss of polar groups and decrease in solubility, while at the antigen excess end the antigen, with more combining sites than antibody, would not permit close-packing (4). The present calculations tend to support and extend this view, for it has been shown that at the antibody excess end the composition of the precipitate in several systems is of the same order as for the Ea system, namely, EaA_4 to EaA_6 (60) (See also table 3). This would involve the close association of relatively many A molecules, especially if

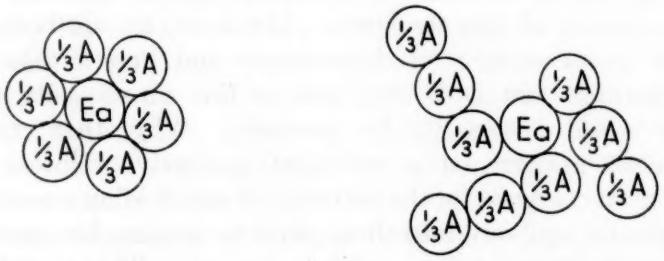
polymerization occurred at the same time in some such way as indicated in figure 2. At the other end of the system, where relatively small, simple, soluble compounds have been shown experimentally to be present (58, 61), a more extended configuration of such molecules can be pictured (figure 2).

ANALYSIS OF OBJECTIONS TO THE QUANTITATIVE THEORY

From the above discussion it will be clear that the conception of specific precipitation and agglutination as the combination of multivalent antigen with multivalent antibody entails as a consequence both the qualitative and quantitative explanation of much that has hitherto been obscure. However, the mere agreement of even large bodies of data with a theory does not guarantee its validity, nor does its serviceability as a tool exclude the possibility that an alternative theory might serve even better. Objections to the qualitative as well as quantitative features of the theory have been made, and these will now be considered.

A sizable portion of the evidence that antigens may possess multiple determinant groups has been supplied by Hooker and Boyd (62). In a discussion leading to the conclusion that the aggregation phase of specific precipitation is non-specific (63) these workers maintain that the assumption of multivalence or multiple determinant groups in the antibody molecule is unnecessary and complicating. However, both antigens and antibodies as proteins possess the same basic structure, and immunological as well as chemical multivalence follows naturally from what is known of this structure. Moreover, an antibody may function as an antigen (cf. Landsteiner and Prasek (64) Ando (65), Marrack and Duff (66)) and in this capacity its multivalence would necessarily be conceded. Why, then, need a multivalent antigen be a univalent antibody? Hooker (67) has, moreover, argued for the necessity of subdividing a combining group on the antibody into three parts to account for specificity differences between *dextro-* and *levo-* isomers. This would seem to imply a limited form of multivalence, for each part would presumably be a chemical bond. Also, in discussing the question

of valence Hooker and Boyd admit (63) the "tacit" assumption of multivalence in calculations relating the antibody: antigen ratios at the equivalence point to the molecular weight of the antigen. But the assumption appears to the writer more than "tacit," and if Hooker and Boyd now believe antibody to be univalent they must abandon the entire basis for these and others of their calculations. For purposes of calculation antibody molecules were assumed by Boyd and Hooker (68) to consist of three (four would perhaps have been better) spherical Svedberg units of 34,500. At the equivalence point (a point definable only as a mean value in an often broad equivalence zone) it was assumed that "the antigen molecule is just completely covered by molecules of antibody" in such a way that "each of the three component units is in contact with the antigenic surface." This, of course, implies at least one combining group on each Svedberg unit, otherwise there would be no reason for the supposedly flexibly joined units to attach themselves to the antigen surface. But it is well known that antigen can combine with at least twice as much antibody as at the "equivalence point." It must then either be assumed that the additional antibody combines with that already on the antigen (and there is no evidence than any part of the antibody is more loosely combined than the rest) or it must be assumed that antibody may also combine head on,[4] so to speak. The two modes of combination might be indicated two-dimensionally as follows for the Ea-A system:



[4] Combination of this type, also, is now proposed by Boyd and Hooker (*J. Gen. Physiol.* 1939: **22,** 281).

The latter alternative is merely another type of diagram such as that given in figure 2, for the A valences remaining free could, and doubtless would, combine with other Ea-A combinations. It also follows from this that the calculation (63, footnote pp. 343, 344) that 15 antibody molecules would more than cover the surface of an antigen the size of the hemocyanin of *C. irroratus* cannot be correct, so that the deduction from this calculation must also be abandoned.

Aside from the valence inconsistency the above hypothesis fails to account for the existence of the whole series of equivalence-zone compounds between EaA_3 and EaA_2, which are often experimentally demonstrable and which are easily accounted for on the basis of multivalent antigen and antibody (cf. also fig. 2). Hooker and Boyd themselves appear to have receded from a too rigid application of their theory of the relation between the molecular weight of the antigen and its combining ratio with antibody at the "equivalence point" (cf. 32, p. 718; also Ref. 57).

The argument for the multivalence of antibody is further strengthened by evidence for the existence of univalent antibody as well. In the papers on the quantitative theory it has been shown that in addition to antibodies which are considered multivalent there is also antibody which behaves as if it were univalent, since when isolated it can no longer combine with antigen to form aggregates, but can only do so when more complete, or multivalent, antibody is present (32). If flocculation is merely a non-specific consequence of antigen-antibody combination due to the presence of salts such antibody should not exist. Its existence, however, has been repeatedly demonstrated, and it has also been shown to combine with antigen (unpublished experiments of the writer).

In support of the non-specific aggregation of the antigen-antibody complex Hooker and Boyd have submitted experiments on the mixed agglutination of red cells. It will be recalled that Abramson (69) had shown that mixtures of red cells and bacteria with antisera to both agglutinated to form mixed clumps and not separate, homogeneous aggregates as would seem to be demanded by the chemical theory, and as was indeed actually shown to

take place by Topley, Wilson, and Duncan (56) in the case of two different bacteria and their antisera. A legitimate criticism of Abramson's experiment would seem to be that the relatively enormous size of the red cells could have blocked the free movement of agglutinating bacteria and so prevented homogeneous agglutination of each cell species. It is also quite possible that the large size of red cells accounts for the mixed agglutination observed by Hooker and Boyd.

Very recently Boyd and Hooker (70) have reported that red cells were agglutinated in the presence of a huge excess of antibody, when presumably every reactive site on the surface was occupied by a molecule of antibody and there appeared to be no opportunity for the formation of aggregates through free antigen linkages. This is taken to indicate non-specific aggregation for this set of conditions. This would, however, be only a limiting case for the theory that aggregation is merely a continuation of the process of combination of multivalent antigen and multivalent antibody, just as the solution of the precipitate, or absence of precipitation in the region of great antigen excess is also a limiting case. As already noted on pp. 71–5, in both instances relatively simple molecules are formed. In the antigen-excess region structural considerations and the molecular ratios of the components favor soluble compounds. In the antibody-excess region symmetry and close-packing (4) favor insolubility. An insoluble *molecule* of EaA_6, for example, would probably combine with similar molecules to build up aggregates through forces which might be termed non-specific, although possibly due in part to the combination of suitable polar groups on colliding EaA_6 molecules to form salt-like linkages much the same as those in any inorganic precipitate. The texture of precipitates produced in this way, is, however, generally very fine, and differs from the form of those in which relatively less antibody is used and antigen valences are left free to combine with bound as well as unbound antibody. Over this, the principal range of the precipitin and bacterial agglutination reactions, the aggregates become larger and larger and more gelatinous as the chemical reactions involving union of multivalent antigen with multivalent antibody

take place in more nearly equivalent proportions, or, in Marrack's terminology, as lattice formation becomes more and more complete. At the other (antigen) end of the range lattice formation also fails, but this is adequately accounted for on the basis of the union of multivalent antigen with multivalent antibody, as already explained. Boyd and Hooker appear willing to concede lattice formation (with univalent antibody?) in the region of antigen excess.

As matters now stand, there is evidence that so-called non-specific factors may determine flocculation or agglutination in the special case in which all antigen valences are occupied by antibody. Over by far the greater part of the reaction range, however, with the exceptions indicated in the first and last diagrams of figure 2 the possibility remains that chemical linkages of partly "coated" antigen molecules occur through antibody molecules, and much has been explained on this basis that cannot be accounted for on the older theory. It would seem reasonable to conclude that such chemical linkages would occur with greater "avidity" when structural and kinetic considerations show them to be possible than would the more vague non-specific linkages.

Hooker and Boyd have also studied the rate of flocculation in the precipitin reaction, and have found that in the region of antibody excess there is a linear relation between the antigen dilution and the time of flocculation (71). Under the assumption that in this range the antigen particles are maximally coated with antibody a simplified form of v. Smoluchovski's equation for slow colloidal flocculation was considered applicable since it resulted in a linear relation. However, inspection of the table (p. 374) shows that much of the linear range in several of the antigen-antibody systems considered lies in the region in which the composition of the precipitate is known to change with the proportions of the components, and hence the antigen cannot be maximally "coated." There is therefore every reason for the belief that ϵ in v. Smoluchovski's theory would be variable, and not constant, as assumed, so that the data do not permit application of the theory except over a much more restricted range. If the linear relation applies, nevertheless, in the region of varying

composition, it is probable that this is due to a balancing of some of the many unknown factors influencing the rate of flocculation. The experiments quoted therefore offer evidence of non-specific flocculation only in the limiting range covered in the work quoted in the preceding paragraph, and this evidence is weakened by the apparent validity of the linear relation in reaction regions in which it cannot have the theoretical significance given.

The increased speed of flocculation demonstrated by these workers in mixtures is less easy to explain away. As admitted by Hooker and Boyd, there was cross-reactivity of related antigens in one-half of the experiments cited in (63) and these must therefore be excluded. But cross-reactivity could not account for the increased speed of flocculation in the other instances, and this was predictable on the basis of non-specific flocculation. A similar effect has recently been observed by Duncan (72) in mixed Ea-serum albumin-antibody systems, but the flocculation rate of mixed agglutinating systems was not increased. Duncan concluded from this that only chemical aggregation is involved in specific bacterial agglutination, but that, in addition, non-specific factors intervene in specific precipitation. Because of the far-reaching analogies between these two immune reactions, this view seems unlikely and would seem equivalent to merely another way of saying that there are still unknown factors which influence the rate of specific precipitation.

Hooker and Boyd also state (63) that the reagglutination experiment discussed in connection with the quantitative theory (pp. 66–70) may be explained by a dissociation of antibody from the initially agglutinated cells, instead of by a reagglutination of the entire mass due to the combination of multivalent S on the freshly added cells with multivalent antibody on the initially agglutinated cells. If by "dissociation" they mean antibody soaked off by the saline in which the initially agglutinated cells were suspended, this was excluded by the conditions of the experiment, for the cells were repeatedly washed until the supernatants no longer agglutinated added homologous pneumococci. If, on the other hand, it is meant that the added cells carry to completion an exceedingly slight dissociation due to any revers-

ibility of reactions [2], [3], etc., the following evidence to the contrary is available: As in the precipitin reaction, the composition of the agglutinated cells is independent of the antibody concentration at equilibrium, so that any change of this kind due to dissociation and reversibility is too slow to measure. Moreover, if there were a redistribution of antibody to include the newly added cells the aggregates formed should be smaller than before, whereas microscopic and macroscopic observations show them to be strikingly larger, as would be expected if the S on the added cells combined with A on agglutinated cells to link masses together.

Although Eagle (73) has accepted the multivalence of antigen and antibody[5] he has raised objections to the view that flocculation is a consequence of such union (74). However, there was no difficulty in interpreting the action of formaldehyde on antibodies, cited by Eagle, in the light of the chemical theory of flocculation, and as this has been discussed fully in a recent publication (35) repetition would seem unnecessary.

Deductions regarding the mechanism of the precipitin reaction have been made by Haurowitz (75, 76) as a result of his studies on arsanilic acid azoproteins and their reaction with antibodies. Many of these deductions seem questionable since they rest on the assumption that the antigens used were well-defined, single substances. The evidence against this, even in Haurowitz's work, is very strong, for in serial additions to excess antisera only 60 per cent and 33 per cent of the antigens added were actually found in the first, and largest, precipitates in two experiments ((76), Tables II and III, pp. 396, 397). The objections raised by Haurowitz against the quantitative theory (76) seem to rest on a misunderstanding, except that the variable composition of the precipitate is ascribed exclusively to the presence of a number of different antibodies. The views expressed regarding the forces responsible for antigen-antibody combination are based on the Breinl-Haurowitz theory of antibody formation (31), modified to take account of the possible ionic nature of at least the initial

[5] In (35) this statement was inadvertently so worded that the acceptance of aggregation as a consequence might also have seemed implied.

combination (18) and additional influences of polar groups indicated by the work of Chow and Goebel (77). Since these matters are also discussed in detail in (4b) they will not be further touched upon here.

Criticism of the quantitative theory has also been made by Marrack (4b, pp. 470, 471). The occurrence of irreversible bimolecular reactions is doubted in systems which we and others have repeatedly shown to be reversible in the sense that the composition of the precipitate may be changed by addition of either component or by alteration of the salt concentration. The inconsistency involved is admitted, but a high degree of irreversibility must be assumed to exist under a given set of conditions, otherwise the effect of concentration of the component in excess would be greater. The real difficulty probably lies in the assumption that antibody may be treated as a single substance, when, as we and others have frequently shown, it is a mixture of antibodies of different reactivities. We have repeatedly called attention to this oversimplification of the quantitative theory as it now stands, but it at least permits many calculations and predictions to be made with accuracy and a certain degree of utility. As already stated (23, 26, 32), the theory was offered, in the realization of many weaknesses, as a temporary expedient which might be useful until antibody possessed of uniform reactivity could be isolated.

With regard to Marrack's other objection regarding the order in which the various bimolecular steps were considered to take place, the same answer may be given; namely, that it was realized that the reaction did not take place in steps, but that for purposes of calculation it was convenient to make such arbitrary subdivision. If some such scheme be envisaged as that in Fig. 2, and the reactions are assumed to involve individual linkages or valences, the reaction order used for the arbitrary "units" originally adopted does not seem so improbable.[6]

It has also been stated by Malkiel and Boyd (57) that the equations of the quantitative theory do not apply in the region

[6] Progress in computations along this line has already been made and should be reported shortly (private communication from Dr. F. E. Kendall).

of antigen excess in the hemocyanin-antibody system. Equation [5] is cited, also the equation

$$\text{mgm. antigen precipitated} = 2R'A - \frac{(R')^2A^2}{\text{antigen added}} \quad \ldots\ldots [8]$$

(equivalent to [5] with S and A transposed), which is said not to apply in the zone of partial inhibition. When these equations were proposed (23) it was expressly stated that they did not apply in these zones. Malkiel and Boyd therefore emphasize what they evidently consider a weakness of the quantitative theory. The writer, however, is inclined to consider as advantageous the division of the precipitin reaction into definite zones which can be delimited experimentally, especially if this permits the application of a theory and consequent calculations and predictions which do not follow from any purely empirical relation. Moreover Malkiel and Boyd also state that equation [8] and the relation derived from it,

$$\frac{\text{antigen pptd.}}{\text{antibody N pptd.}} = 2R' - \frac{(R')A}{\text{antigen added}} \quad \ldots\ldots [9]$$

which is linear with respect to $\frac{1}{\text{antigen added}}$, do not apply to the hemocyanin system, but insufficient data are given to permit a test of this assertion. The necessary figures were most kindly sent to us and equation [8] (hence [9]) was found to fit for the entire region of maximum antibody precipitation in three out of the four instances tested. We have, then, for this portion of the reaction range, our own equation, derived from the law of mass action, and the empirical relation of Malkiel and Boyd. As for the inhibition zone, many of the figures in the tables given by these workers (57) clearly indicate that the linear relation can be extended into the inhibition zone only with a sharp inflection horizontally. Thus in Table II p. 387, R in the two inhibition-zone precipitates is patently constant for Serum 926_2; R is also constant, within the large experimental error, for the three inhibition-zone precipitates of Serum 928, Table V, p. 380, and for the three precipitates in this zone from Serum 928_2, p. 381.

In three of the six instances cited, then, the data do not warrant the extension of Malkiel and Boyd's empirical relation into the second zone to which it is said to apply. In two other antigen-antibody systems for which our own data are sufficiently accurate, two out of four sera studied have shown constant composition of the precipitate in the zone of partial inhibition ((23), p. 567; (32), p. 235). It may be pointed out that precipitates of constant composition in equilibrium with the soluble inhibition-zone compound are entirely consistent with the theory of the union of multivalent antigen with multivalent antibody.[7]

While the above detailed discussion of the precipitin reaction and specific bacterial agglutination may have seemed overlong, these two immune reactions are the only ones for which a considerable body of precise, absolute data exists. Since this review concerns itself primarily with such data these two reactions have necessarily taken up most of the space allotted.

THE TOXIN-ANTITOXIN REACTION

A beginning has been made, however, toward placing another of the most important immune reactions on a similar basis. Until recently the voluminous knowledge of the toxin-antitoxin reaction could be expressed only in relative terms such as were

[7] We have had the privilege of discussing this matter with Mr. Malkiel and Dr. Boyd and have sent them this paragraph for further comment, which is appended herewith.

Rejoinder by Mr. Malkiel and Dr. Boyd: We realized that [5] was not intended to apply to the region of antigen excess, nor [8] to the inhibition zone, but did wish to emphasize what still seems to us a weakness in the theory, which accounts for the precipitin reaction quantitatively only by dividing it into three regions, with a different equation for each. We regret not including enough auxiliary data to enable the reader to try [8]. We meant to say [8] did not apply in the inhibition zone ("large antigen excess"), and pointed out this was evident from its form, without numerical test. To us, the zone of antigen excess and the inhibition zone seem continuous and essentially similar. We feel that the fit obtained with some of our data and [9] is simply that always possible if a curvilinear relation is tested against linear data for a relatively small portion of its mathematical range; our experimental errors are admittedly relatively large. If our empirical relation is really a straight line, it is impossible that [9], which, instead of R (our symbols), contains 1/R, should also be straight, if plotted similarly.

formerly used for the precipitin and agglutinin reactions, and for the same reasons. With the isolation of what is presumably pure diphtheria toxin by Eaton (78) and by Pappenheimer and Johnson (79) the antigen has become known as a protein, the properties of which may be followed quantitatively. In the flocculation zone of the reaction the methods of analysis developed for the quantitative study of the precipitin and agglutinin reactions have also been found applicable.

The first study of this nature was made by Marrack and Smith (80), who showed that diphtheria toxin-antitoxin floccules were mainly "denatured" pseudoglobulin, and that the amount of nitrogen precipitated was independent of the quantity of non-specific serum proteins present and increased with increasing amounts of antitoxin (A) up to the flocculation limit. In sera showing an *in vivo: in vitro* ratio of 1 or greater, Healey and Pinfield (81) found that if the composition of the toxin-antitoxin floccules were represented as TA (in units) at the Ramon flocculation point, the composition TA_2 could be attained when A was present in twice the amount. Over all but the ends of the flocculation range all of the T and A present were in the precipitate. It was also found that TA floccules could combine with A or a relatively small amount of T, and that TA_2 floccules combined with T, but with very little A. The reversibility of the Danysz effect (cf. also (82, 83) was demonstrated.

A more detailed quantitative study was made by Pappenheimer and Robinson (83), using highly purified toxin, and in some instances purified antitoxin. It was found that the flocculation zone corresponded to the equivalence zone of precipitin reactions in which neither component is demonstrable in the supernatants, in accord with Healey and Pinfield. The nitrogen figures cited indicate approximately a three-fold range of combining proportions over the entire zone. Since the Danysz effect is shown outside the zone of flocculation, where any effect due to the combination of T and A in varying proportions should be immediately reversible, it is postulated that T and A combine rapidly to form a soluble compound, followed by a slow reaction resulting in flocculation when the proportions are suitable. The initial

rapid reaction would be responsible for the Danysz effect and this would be slowly reversible after addition of the last portion(s) of T.

The amount of N per Lf unit of T was calculated by subtracting the N precipitated from 300 units of A by 200 Lf of T from that precipitated by 400 units. In six sets of determinations the values ranged from 0.00042 to 0.00048 mgm. of N, with a mean of 0.00046, regardless of the purity of the T or A used, and agreeing with the value given by Eaton (78) for his purest T. With this value it is shown that a constant figure is obtained for the A precipitable throughout the equivalence zone except with A showing evidences of alteration. The value found was, in general, 0.016 mg. of N per flocculation unit of A. Using these figures, the A:T (or more strictly, A N:T N) ratios at the A-end, flocculation point, and T-end of the equivalence zone were found to be approximately 7.0, 3.5, and 2.5, respectively. With the aid of the flocculation-point ratio and a single pair of duplicate nitrogen determinations the potency of both an unknown toxin and an unknown antitoxin may be calculated, even if no standard is available for comparison!

THE RÔLE OF LIPIDS

Quantitative analytical methods both for antibody and lipids have been used by Horsfall and Goodner (84) in studying the relation of lipids to specific precipitation and agglutination in antipneumococcus sera. Of the many significant observations and analyses made only those will be discussed bearing directly on the mechanism of the precipitin reaction. It was found that the first precipitate formed carried down a relatively high proportion of the lipid present, and lipid contents as high as 51 per cent were noted. Subsequent precipitates in the same serum were relatively low in lipid and the amount in any case showed no relation to the nitrogen (protein) content. Thorough extraction of the lipids abolished specific precipitation and agglutination in the case of horse serum, and greatly diminished these effects in rabbit serum, in line with the earlier work of Hartley (85),

but lecithin restored these properties to extracted horse serum and cephalin to extracted rabbit serum. It was considered that most of the lipid carried down was adsorbed to the specific precipitate, but that the exceedingly small amount of lecithin or cephalin necessary to restore the precipitating and agglutinating power might indicate that antibody consisted of a lipo-protein complex. Highly purified antibody from horse serum was also stated to contain lecithin (84d). It was, however, found that the extracted antibody could still combine with pneumococci, although these were not agglutinated, so that an alternative hypothesis seems to the writer at least equally probable. The observation, quoted above, that the first precipitate in a series contains relatively much lipid, and that subsequent precipitates contain less lipid (and often form more slowly) might be taken to indicate that the function of lipid in promoting specific precipitation and agglutination is essentially a mechanical one. The effect of the lipid would then be to provide nuclei for the formation of aggregates, much as dust particles in a supersaturated solution of a substance promote its crystallization.

In the foregoing review immune reactions involving specific precipitation and bacterial agglutination have been discussed in the light of newer data obtained by quantitative methods conforming to the criteria of analytical chemistry. An outline has been given of the progress made, since the introduction of these methods, toward the understanding of these immune reactions, and instances have been given of the utility of the methods and the theories based on them. The analytical methods have withstood all tests for accuracy and reliability and are now quite generally accepted as standard, but the quantitative theory, in spite of its utility, is obviously defective in certain respects. It is hoped that this discussion of the present state of these problems will stimulate not only the search for more rigorous theoretical explanations than are now available, but will also further the application of suitable absolute methods to other, more intricate manifestations of immunity.

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THE PATHOGENIC STAPHYLOCOCCI

JOHN E. BLAIR

Laboratory Division, Hospital for Joint Diseases, New York

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CONTENTS

During the past several years the pathogenic staphylococci have received considerable attention, particularly with regard to their toxigenic capacities and the relation of their toxins to staphylococcal infections. Papers between 1924 and 1931 by Parker (201, 258), Gross (102–110), and Burnet (26–28), and by many others in the years immediately following, have yielded new information, have established a new basis for the interpretation of known facts about staphylococci, and occasionally have reëmphasized earlier observations upon staphylococci.

Burnet's study of these organisms followed an incident in Bundaberg, Queensland, in 1928, which demonstrated the toxi-

genic potentiality of staphylococci. Of twenty-one children who received a routine prophylactic injection of diphtheria toxin-antitoxin, twelve died within 34 hours with acute staphylococcal toxemia (144). A culture of the toxin-antitoxin yielded *Staphylococcus aureus.*

A brief review of some of the newer information was made by Holman (126) in 1935. Considerable material has continued to appear, and it has seemed advisable to summarize some of the contributions to the study of staphylococci that have been made during the past decade or so. Such a summary must necessarily be limited in scope in this review; the immunology and therapy of staphylococcal infections must be omitted. A limitation is also imposed by the very newness of some of the material, which will acquire significance only through the perspective to be gained by the solution of present problems. However, it is to be hoped that a summary at the present time will serve to emphasize certain salient features of recent investigations, and will afford points of departure for future work.

EXOTOXIN

Definite demonstrations of the production of toxins by staphylococci were made by the earlier investigators (159, 190, 195, 229, 251). These reports received only passing attention, however, and little emphasis was subsequently given to the toxigenic capacities of staphylococci, or the possible rôle of toxins in the pathogenesis of staphylococcal infections. From the more recent information it is now firmly established that certain strains of staphylococci produce a soluble exotoxin, which may be obtained in culture filtrates, and which is quite comparable to other recognized bacterial toxins in its antigenicity and other properties.

In accordance with the effects produced by potent culture filtrates, various self-descriptive terms have been employed, *viz.*, lethal toxin, dermonecrotoxin (or dermotoxin), hemotoxin, leucocidin, enterotoxin, and coagulase and fibrinolysin. This variety of effects raises the question whether some of them may be due to a single toxin whose action varies with the cells with

which it comes in contact, or whether each toxic effect is due to a separate toxic factor. Regardless of the ultimate solution of this question, the terms at present supply a convenient nomenclature in the discussion of the activity of pathogenic staphylococci.

Under the heading "exotoxin" is to be described the soluble antigenic toxic substance produced by many pathogenic staphylococci. Toxic filtrates of staphylococcal cultures produce death (from lethal toxin) or dermonecrosis (from dermonecrotoxin) when injected into experimental animals in suitable doses and by the proper routes. Since hemolysis of rabbit erythrocytes is considered by many as another manifestation of the action of exotoxin (26, 28, 86, 115, 192, 217–223), a discussion of "hemotoxin" may for convenience be included under the general heading of exotoxin, subject to such reservations as will be indicated below.

Exotoxin in varying amounts appears to be produced by many, but by no means all, pathogenic staphylococci. From 85 to 90 per cent of strains studied by Dolman (60) and by Stookey and Scarpellino (237) were reported to produce demonstrable amounts of toxin. Our own unpublished findings are quite comparable to these. However, it appears that a much smaller proportion of strains produces a potent lethal toxin (24, 192, 229).

Preparation of staphylococcal toxin

In the study of staphylococci attention has necessarily been paid to the determination of the optimum conditions for the formation of their toxins. In many laboratories at present culture media are used that are based ultimately upon those described by Walbum (255) and by Parker (201). Essentially the method now in common use is to grow staphylococci in broth or on a semisolid medium (containing less than one per cent agar) with a veal- or beef-infusion base, in an atmosphere of 20 to 40 per cent CO_2, for one to three days, or occasionally longer. Modifications which have been adopted in various laboratories generally involve slight alterations in the amounts of various ingredients, the reaction of the medium, and methods

of harvesting and preserving the toxins. The composition of the medium appears to be of considerable importance, and its modification may be a source of confusion in the interpretation of results (25, 207).

Valuable information has been obtained concerning the nutritive requirements of staphylococci. Following the demonstration by Hughes (130) and by Knight (153) of growth-accessory factors, subsequent work has shown that nicotinic acid or nicotinamide and thiamin (vitamin B_1), effective in minute amounts, are required by the staphylococcus for its growth (71, 118, 125, 154, 155, 158, 161). The requirement for these factors is highly specific, for even closely related compounds are ineffective in promoting growth (155, 156, 161). Richardson (216) has shown that uracil is necessary for the anaerobic growth of staphylococci, its requirement also being highly specific.

It is apparent that the use of synthetic media for the production of toxins would be desirable, for it would allow a more accurate study of the nature of staphylococcal toxin than has yet been possible. Staphylococci will grow satisfactorily in an entirely synthetic medium containing the growth accessory factors (71), and recently Gladstone (89) has been able to obtain good toxin in a medium of known chemical composition. Media of somewhat simplified composition have been employed for the successful production of the toxin. Leonard and Holm (163) have described a "semi-synthetic" medium, which is used in an atmosphere of 80 per cent CO_2. Media prepared with the dialysate of nutrient broth have been used by Holt (127) and by McClean (188). These possess the advantage of containing distinctly less nitrogenous material than the whole broth, and the toxins obtained appear to be antigenically equal to those obtained by the methods more generally employed.

The introduction of the use of semisolid agar (27) and of an atmosphere of partial CO_2 tension (203) represent distinct additions to the methods of preparation of staphylococcal toxin. Birch-Hirschfeld (8) obtained potent toxin by growing staphylococci on the surface of cellophane on agar plates. She attributed this to autolysis of the cocci by metabolic products which did

not diffuse through the cellophane. It has been shown by McClean (188) that the action of cellophane, of agar, and of some other materials, is to adsorb from the medium a substance, as yet unidentified, which inhibits toxin-formation.

While the presence of CO_2 may directly stimulate growth of the cocci, as it is known to do in the case of certain other bacteria, its chief advantage appears to be its action in maintaining the reaction of the medium at a level which best favors the formation of toxin. Bigger (6) reported that CO_2 is definitely prejudicial to the formation of toxin by an occasional strain of staphylococcus. To prevent excessive alkalinization, Bigger adopted a combination of glycerol and a phosphate buffer. Apparently a similar effect was obtained by Nélis, Bouckaert, and Picard (192), who reported that 0.1 per cent of glucose favored toxin-production.

The possible association of oxidation-reduction processes with the production of staphylococcal toxin is suggested in recent work by McBroom (185). She has noted that the production of hemotoxin by staphylococci is definitely correlated with their ability to reduce methylene blue. No such correlation was found with their proteolytic, lipolytic, or carbohydrate-utilizing capacities. Burky (21) has reported that a toxigenic strain of staphylococcus when grown anaerobically produced lethal toxin, but no hemotoxin.

Properties of staphylococcal exotoxin

Like some other bacterial toxins, staphylococcal exotoxin is readily thermolabile. Loss of toxicity is generally reported to occur between 55° and 60°C., but no reference is made to the effect on antigenicity (57, 158, 258). The toxin is sensitive to light and oxidation. Li (166, 167) has reported the complete destruction of the toxic properties (lethal, necrotic, hemolytic) by the action of light and methylene blue, with no loss of antigenicity.

The concentration and purification of toxin was attempted by Burnet and Freeman (28), who used the methods often applied to other bacterial toxins. By precipitation with acetic acid a

yield of from 60 to 90 per cent was obtained, much of the nitrogenous matter of the crude toxin being removed. Precipitation with trichloracetic acid was successfully employed by Boivin and Izard (14). Reference has been made above to the use of a dialysate medium by Holt and by McClean for the production of toxin free from the non-dialyzable nitrogenous material of the whole broth.

Ultracentrifugation of staphylococcal hemotoxin by Gratia and Nélis (101) resulted in a fluid the upper layers of which contained the toxin, and the lower layers none. There was, however, no appreciable concentration of the toxic factor.

Although exotoxin is filtrable, much of it may be lost by adsorption on certain types of filter (229, 262). Woolpert and Dack found that lethal toxin, dermonecrotoxin, and hemotoxin were retained to a great extent by Seitz filters, while Berkefeld "N" candles yielded filtrates of high potency (262). This is comparable to similar findings for staphylococcal leucocidin (248) and for staphylocoagulase (72).

Nélis, Bouckaert, and Picard (192) reported that in equal concentrations mineral acids destroy the toxin, while organic acids vary in their action, from partial destruction to none at all. The speed of the reaction had a direct relation to the temperature. Colloidal iron and manganese have been reported to inhibit lethal toxin, while colloidal gold, silver, and platinum had no effect (162). Neither of these reports refers to the effect of these agents upon antigenicity.

Llewellyn Smith (169) has recently reported that glycerol, ethylene glycol, sucrose, and glucose inhibit the dermonecrotic action, due to partial destruction of the toxin. Experiments to determine the effect on antigenicity were inconclusive. The lethal toxicity for rabbits was also significantly diminished, but the hemolytic action was unaltered. In the latter instance this was probably due to the lower concentration of glycerol used in hemolytic tests, for in equal concentrations glycerol inhibited hemolysis and dermonecrosis in parallel fashion. The effect of glycerol should be remembered when the dermonecrotic test

is used to titrate antitoxic sera that have been preserved with glycerol.

Rigdon and his associates (4, 218, 221) found that hemotoxin and dermonecrotoxin were inhibited by hypertonic solutions of sodium chloride, and by certain other salts of sodium, potassium, and magnesium. This was attributed to some action of the salts on the cells which rendered them refractory to the action of the toxin. Lithium chloride also inhibited dermonecrosis but had no effect on hemolysis. Weinstein (257) reported that lecithin inhibited hemolysis in blood agar by staphylococci and streptococci, and that cholesterol exerted an antagonistic action, preventing the inhibition by lecithin. The lethal, necrotic, and hemolytic capacities of toxin were reported by Schwartz (230) to be considerably reduced by olive oil.

Staphylococcal Toxoid (Anatoxin)

The effect of a suitable concentration of formaldehyde is well known, since it is the accepted agent for detoxifying toxin in the preparation of toxoid. In a concentration of 0.3 to 0.5 per cent complete detoxification is accomplished at 37°C. in from 7 to 20 days. The lethal, dermonecrotic, and hemolytic properties are destroyed, without loss of the antigenicity. In concentrations up to one per cent, more rapid detoxification, accompanied by greater antigenicity, has been reported by Kitching and Farrell (151). Burnet and Freeman (29) found that the rate of detoxification bears a direct relation to the concentration of hydroxyl ions and possesses a high temperature coefficient.

The immunizing power may be enhanced by precipitation of toxoid with alum (69, 163), with saturated ammonium sulfate (127), or with trichloracetic acid at pH 4.0 (209). The alum-toxoid is particularly effective as a primary stimulus in immunization (69).

Tests for antigenicity of toxoid involve the determination of binding and flocculating properties, and animal inoculations. The biological standardization offers certain difficulties, which have been discussed by Llewellyn Smith (168). Factors which

are involved include dosage and time intervals, great individual variation in response to the antigen, the effect of diet and seasonal variations, and differences in response to various types of antigen, such as toxoid, alum-toxoid, and toxin-antitoxin or toxoid-antitoxin floccules. However, Dolman and Kitching (62) regard the biologic method as of only qualitative value for toxoids of low immunizing efficacy. Kitching and Farrell (151) have reported a rough correlation of binding power and the response *in vivo* to antigenic toxoid preparations.

Antigenicity of staphylococcal toxin and toxoid

The possibility of producing antitoxin in high titer by a suitable course of immunization was demonstrated in laboratory animals by Parker (201), Burnet (26), Dolman (57), and Gross (109), in horses by Parker and Banzhaf (202) and by Burnet (28), and in human volunteers by Dolman (57). In reports supplying repeated confirmation of this, the experimental production of complete antitoxic immunity has sometimes been claimed, and the protection thus established was frequently related to an increased titer of circulating antitoxin. A basis was thus established for the therapeutic use of toxoid and antitoxin.

Conflicting reports of the therapeutic value of staphylococcal toxoid and antitoxin are found in the literature. While certain types of staphylococcal infection appear definitely to benefit from this type of therapy, some unfavorable results may be attributed to uncritical selection of cases, and also to a premature conception of the part played by toxin in the pathogenesis of certain staphylococcal infections.

The serologic problems raised have necessitated a common basis for the comparison of results. Under the direction of the Committee on Standards of the League of Nations an international standard staphylococcal antitoxin has been established (115). Antisera are titrated for their ability to inhibit hemolysis, dermonecrosis, or the lethal action of toxin.

THE ACTION OF STAPHYLOCOCCAL EXOTOXIN

Laboratory animals are susceptible in varying degrees to the lethal effects of the toxin. The rabbit is particularly susceptible,

and usually is the animal of choice. Wild rabbits used by Kellaway, Burnet, and Williams (143) were reported to be five times more susceptible than the domestic varieties. Guinea pigs are definitely less susceptible than rabbits, as is shown by the relatively larger effective dose required for lethal or dermonecrotic tests (27, 60, 162). Adult animals, both rabbits and guinea pigs, appear to be relatively more susceptible than young animals (23, 57, 162), although young animals have sometimes been used (262). Cats, dogs, monkeys, mice, rats, horses, and doves show varying susceptibility (57, 60, 143, 217, 229, 262). The toxin is administered intravascularly. There appears to be good evidence that when given by mouth, exotoxin is without effect (60).

In considering the lethal potency of staphylococcal toxin, it must be remembered that the toxins studied to date generally represent relatively crude filtrates of cultures. Consequently the figures for the minimal lethal dose do not approach the order of those reported for some other toxigenic bacteria. The M.L.D. of staphylococcal toxin, following injection into the blood stream of rabbits, has generally been reported as between 0.1 and 0.5 ml. per kilogram of body weight (21, 26, 57, 60, 143, 163, 199, 262). Roy has recently recorded lethal titers ranging down to 0.0016 ml. per kilogram (228).

It has been well established that the ability of a strain of staphylococcus to produce exotoxin is in no way related to the severity of the infection from which it was isolated (57, 192, 198, 200, 232). A similar lack of relationship was reported by Minett for strains from veterinary sources (182).

The lethal action of toxic filtrates

A striking series of events follows the intravenous injection of a potent staphylococcal toxin into a susceptible animal (26, 57, 60, 143, 195, 217, 229, 262). The reaction may be immediate (death resulting in five to fifteen minutes), or it may be delayed (death occurring in two to four hours, and more often in twenty-four hours or longer). For a brief interval the animal appears to be normal. Then, in rapid sequence, it becomes unsteady and paralysis of the hind legs develops; the respiration, which may

be rapid at first, becomes irregular and gasping; incoördinate running movements often occur; initial contraction of the pupils is followed by wide dilatation. The animal then usually dies after violent convulsions, or it may become moribund until death supervenes. There is often incontinence of urine and feces, and sometimes diarrhea.

When death is delayed for a few hours, a somewhat similar series of events occurs, but more slowly. Opisthotonos, and a marked loss of weight (sometimes as much as 200 grams per day) have been recorded by Burky (21) in rabbits surviving for more than twenty-four hours. In cats, vomiting was described in the earlier stages by Kellaway, Burnet, and Williams (143). It is significant that the symptoms exhibited by the children who died of staphylococcal toxemia at Bundaberg were quite similar to the experimentally produced reactions described above.

Investigating the mode of death, Kellaway, Burnet, and Williams showed that in addition to a direct action on the heart (an effect sometimes preventable by active or passive immunization), the toxin also affects the vascular supply of the lungs (143). This results in obstruction of the pulmonary circulation, with consequent acute failure of the right heart. An initial transient fall of blood pressure occurs, apparently due to pharmacologically active constituents of the culture medium. This is followed by recovery to or above normal with maintenance of the blood pressure at about the normal level. An increased output of adrenalin aids this recovery (52, 143). Finally there is a characteristically rapid terminal fall of blood pressure, occasioned by failure of the pulmonary circulation (143).

The possibility has been considered that histamine in the toxin might be responsible for the characteristic rapid death of experimental animals (26, 57, 219); but this has been dismissed by Burnet (26) and by Dolman (57). More recently Feldberg and Keogh (70) have reported that histamine is liberated during perfusion of the guinea pig's and cat's lung with toxin. They suggested that this might explain the acute fall of arterial pressure, rise in pulmonary pressure and peripheral vasodilatation.

Several more or less detailed studies have been made of the pathologic changes which follow the intravenous administration

of exotoxin (16, 52, 90, 92, 143, 192, 217, 219, 225, 226). The chief features that are seen in the gross and confirmed microscopically are serous or serosanguinous exudate into the pericardial, pleural, and peritoneal cavities, congestion of various organs, and a characteristic finding of hemorrhages, petechial or extensive, in the organs and serous linings of cavities. The majority of blood vessels are dilated, and intravascular hemolysis, which may be quite intense, is frequently found (143, 192, 261). Injury to the tissues is indicated by changes which vary from cloudy swelling to intense necrosis.

The dermonecrotic action of toxic filtrates

The necrotic action of staphylococcal toxin is exemplified by the reaction of dermonecrosis. This was carefully studied and described by Parker (201), and has received repeated confirmation by others. Dermonecrotoxin appears to parallel the lethal toxin in its production, properties, and antigenicity. The reaction appears frequently to serve as well as the lethal action in determining the toxic effect of potent filtrates. The general description of exotoxin given above applies essentially also to dermonecrotoxin, and requires no further elaboration at this point.

The hemolytic action of toxic filtrates

In addition to their lethal and necrotic properties, many toxic staphylococcal filtrates are also capable of hemolyzing erythrocytes. Evidence obtained from tests for hemolysin and neutralization of hemolysin by antiserum suggested the existence of more than one hemolysin (or hemotoxin). Glenny and Stevens (91) have demonstrated that there are at least two antigenically distinct hemotoxins, which they have designated as "α-toxin" and "β-toxin." This has received ample confirmation by Bryce and Rountree (19) and others. The commonly described staphylococcal hemolysin is α-hemotoxin, active at 37°C. on both rabbit and sheep erythrocytes, and frequently characteristic of pathogenic staphylococci from human sources. The β-hemotoxin is inactive on rabbit cells, but is active against sheep cells, and then only after incubation at 37°C., followed by incubation at

a lower temperature. It does not appear to be related to human pathogenicity. Although produced by some strains of human origin, β-hemotoxin appears to be quite characteristic of veterinary staphylococci (19, 182). These strains usually produce both α- and β-toxins, but some strains produce β-toxin almost or entirely to the exclusion of α-toxin.

Morgan and Graydon (183) have demonstrated the existence of two α-hemotoxins, which they call "α_1-toxin" and "α_2-toxin." The latter was usually present in only small amounts in a toxic filtrate. Flaum and Forssman (76) noted the distinction between lysins for rabbit and sheep cells, and suggested that α-toxin was a mixture of two lysins. It is possible that one of these corresponds to the α_2-toxin of Morgan and Graydon.

The β-hemotoxin appears to correspond to the "hot-cold" lysin studied by Bigger (6) and Bigger, Boland, and O'Meara (7), and previously reported by Walbum (254). Roy (228) and Flaum (75) have suggested that the lysin active on human erythrocytes is identical with β-toxin, but Roy's human lysin also hemolyzes rabbit erythrocytes.

The physical properties and antigenicity of α-hemotoxin are quite similar to those of exotoxin. In contrast, the β-hemotoxin appears to be thermostable (6, 7, 19, 263), although Minett (182) reported little difference in resistance to a temperature of 55°C. An antigenic toxoid was prepared by Bryce and Rountree (19) by the action of formaldehyde on β-toxin. Levine (164, 165) has suggested that the combination of red blood-cells and lysin may be due to adsorption, for the reaction was found to conform to the general principles controlling adsorption phenomena.

The susceptibility of the erythrocytes of various animal species to α-hemotoxin is of interest, and considering the variations in technique among different laboratories, the broad general correspondence is striking. The following table lists in decreasing order the sensitivity of various mammalian erythrocytes to α-hemotoxin:

Bryce and Rountree (19): Rabbit, Ox, Koala, Sheep, Ferret, Rat, Human, Guinea pig.

Dolman (57): Rabbit, Sheep, Cow, Guinea pig, Human, Cat, Horse.
Forssman (77): Rabbit, Ox, Sheep, Goat, Human, Horse,[1] Guinea pig.[1]
Gross (107): Rabbit, Cow, Sheep, Goat, Human, Horse, Guinea pig.
Le Fèvre de Arric (162): Rabbit, Guinea pig.
Minett (182): Rabbit, Ox, Sheep, Dog, Human.
Woolpert and Dack (262): Rabbit, Mouse, Dog, Monkey, Rat, Sheep, Guinea pig, Human.

Regarding susceptibility to β-toxin, bovine (19, 182) and human (75, 228) red cells have been reported to rank close to sheep cells. In comparison to sheep cells, Bryce and Rountree recorded the susceptibility to β-toxin of the red cells of several other species in the following approximate decreasing order: ox, human, koala, ferret, guinea pig, rabbit, and rat. Except for the ox cells, which were about as sensitive as sheep cells, all of these showed little or no hemolysis. Minett (182) found that the erythrocytes of the dog and horse were relatively resistant to β-toxin.

It would appear that hemolysis in blood agar should not be considered as any more than suggestive of the production of hemotoxin, and certainly the use of blood-agar plates does not lend itself to a quantitative interpretation. It is well known that hemolysis in blood-agar is not necessarily related to the production of soluble hemotoxin (243, 244). Hallman (114), in this laboratory, has reported that 91.4 per cent of 480 strains of staphylococci from nasal mucous membranes hemolyzed human blood-agar. Only 67 per cent of these strains were potential pathogens. Comparable results were obtained recently by McFarlan (189).

Correlation of the toxic factors

The belief has been expressed by a number of authors that lethal toxin, dermonecrotoxin, and α-hemotoxin are probably identical (26, 28, 86, 115, 192, 217–223). Claim for the unity of the toxins was based upon similarity of physical properties, and upon the fact that all three were found in quantitative relationship in the filtrates studied, and were quantitatively neutralized by a single antiserum. When quantitative differences

[1] No hemolysis.

between the toxic factors of certain filtrates occurred, they were attributed to the destruction or attenuation of one or more of the toxic functions of these filtrates, possibly in the "toxophore" group of antigenically identical antigens (26, 29).

On the basis of adsorption experiments with erythrocytes the identity of hemotoxin with lethal or dermonecrotoxin has both been claimed (86, 192) and denied (258). Adsorption tests performed by Roy (228) were inconclusive, but suggested a relationship between hemotoxin for sheep and human cells, and a difference between this lysin and that active on rabbit cells. The results of all of these experiments were rarely clear-cut, and at best are only suggestive of the relationships claimed.

Gengou (86) has suggested that, rather than the constant presence of the three toxins in filtrates to indicate unity, the absence of one of them from given filtrates would be better proof of their lack of identity. This condition appears to have been realized in some of the filtrates described by Parker (201), Burky (21, 24), and Flaum and Forssman (76). Parker reported that many of her "non-poison-producing strains" (which produced no dermonecrotoxin) were strongly hemolytic, and that filtrates containing potent dermonecrotoxin were usually ineffective when injected intravenously into rabbits. Flaum and Forssman studied antigenic hemolytic filtrates which were neither lethal nor dermonecrotic. Burky reported the production of lethal toxin, but no hemotoxin, anaerobically in hormone broth and aerobically in Uschinsky's medium. Studying a strain which originally produced both lethal and hemolytic toxins, he found that after two years' maintenance of the stock strain on artificial media, its ability to hemolyze had almost completely disappeared, and coincidentally its toxicity had increased. The implications of the results of Parker, of Flaum and Forssman, and of Burky should receive serious consideration.

LEUCOCIDIN

It is of historical interest that the earliest description of a bacterial leucocidin was that of the staphylococcus, being described in 1894 by Van de Velde (251). The significance and

probable importance of leucocidin in staphylococcal infections has been repeatedly suggested, and tacitly assumed, but, as pointed out by Holman (126), technical difficulties have hampered its study. A common method of approach has been by means of the familiar "bioscopic" test, introduced by Neisser and Wechsberg (190). Recently Valentine (248) has employed a microscopic method, observing the destruction of leucocytes directly in a series of slide preparations stained with a suitable blood stain.

Valentine's work, which has been confirmed by Proom (207), indicates that the action of leucocidin must be distinguished from that of α-hemotoxin. According to Valentine, "true" staphylococcal leucocidin destroys both human and rabbit leucocytes, but has little or no effect upon the erythrocytes of either species (248). On the other hand, α-hemotoxin has essentially no effect on human red or white cells, but destroys both red and white cells of the rabbit. The microscopic picture differs according to the destructive agent. Human and rabbit leucocytes in contact with leucocidin become spherical, the nuclei fragment, and the granules are arranged circumferentially in the cell. The leucocytes may eventually burst, and this invariably occurs when a dried stained film is prepared. Rabbit white cells destroyed by α-hemotoxin retain the granules grouped in one part of the cell, and remain intact even in the dried film.

It thus appears that these differences in technique have led to differences in interpretation of the exact nature of the leucocidin. It may readily be understood why those investigators using the bioscopic method have considered leucocidin and α-hemotoxin as identical, with similar properties and endpoints of titration (106, 263). Some differences in properties, and an entire lack of correlation of hemolytic and leucocidic titers have been reported following the use of the microscopic method (197, 207, 248).

The differences in technique must be kept in mind in evaluating reports upon leucocidin. Proom (207) has emphasized that the cultural method of preparing the leucocidin affects the significance of bioscopic titrations. It would appear that the Neisser-Wechsberg technique would be applicable only to the

titration of filtrates containing no appreciable amount of α-hemotoxin. For the titration of hemolytic filtrates, Valentine's method, using human cells, is indicated.

It has been generally assumed that the majority of pathogenic staphylococci produce leucocidin. Production of leucocidin has been reported in 17 of 22 strains by Panton and Valentine (197), and in 27 of 36 strains by Valentine (248). All strains in both series were from pathologic sources. All of 26 pathogenic strains were reported by Wright (263) to produce leucocidin (bioscopic method), while 7 strains from non-pathologic sources produced none. Leucocidin titers have been variously reported as ranging from 0.01 to 0.0007 ml. (106), and from 1:200 (263) to 1:500–1:1000 (55, 248).

Leucocidin is antigenic. The production of antileucocidin in rabbits was reported by Denys and Van de Velde (55), Gross (106), and Neisser and Wechsberg (190); its production in horses was described by Panton, Valentine, and Dix (198) and by Wright (263). Titrations of antileucocidin have presented serious technical problems, particularly when attempted by the bioscopic method. No accepted standard for antileucocidin is available, making it necessary for individual laboratories to adopt standards of their own.

A phagocyte-depressing substance produced by staphylococci has been described by Pike (205), in confirmation of earlier work by Hektoen (117) and by Wadsworth and Hoppe (253). It differs from leucocidin in its properties; it is nonspecific, nonantigenic, and cannot be considered as being identical with leucocidin.

COAGULASE

The ability of staphylococci to coagulate blood plasma has been recognized and studied at intervals since the demonstration by Loeb (171) in 1903 that broth cultures of *S. aureus* were capable of coagulating goose plasma. The development of a clot in blood broth cultures from patients with staphylococcal septicemia is familiar to all clinical bacteriologists. The relationship of coagulase to pathogenic staphylococci was noted by Much (184),

and its value in differentiating between pathogenic and nonpathogenic staphylococci has been suggested by several investigators (34, 44, 50, 102, 147).

Among the pathogenic cocci, the ability to coagulate plasma is confined to the staphylococci. A variety of other bacterial species has been found not to coagulate plasma (44, 72, 106), although occasional strains of *Pseudomonas aeruginosa*, *Serratia marcesens*, *Escherichia coli*, and *Bacillus subtilis* have been reported to give a positive reaction (72, 171). Strains of both *S. aureus* and *S. albus* will coagulate plasma, although the coagulating power of *S. albus* strains, as a group, is less marked than that of the *S. aureus* strains (27, 34, 72, 102). The plasma-coagulating property of staphylococci appears to be quite stable, and to be retained longer than such properties as chromogenesis and production of hemotoxin (11, 35, 44, 113).

In general, rabbit or human plasma is most readily clotted, one or the other being sometimes reported as being more satisfactory (44, 94, 256), or both being found to be equally good (72). Occasionally plasma from other species, particularly dog plasma, may be most readily clotted by certain strains (100, 104, 204). In the coagulase test, any amount of clotting is considered positive. There is no relation between the rate of clotting and the virulence of the strain.

The presence of coagulating substance in filtrates, originally denied by Kleinschmidt (152), was demonstrated by Gross (103, 104), and has recently been observed by Fisher (72), Cruikshank (44), and Walston (256). Not all filtrates are active, although coagulation may be obtained with broth or agar cultures of strains yielding inactive filtrates.

A distinguishing feature of coagulase is its resistance to heat, exhibited both by cultures and filtrates (72, 108, 256). First noted by von Gonzenbach and Uemura (94), the later report by Gross (105) that it resisted heating to 100°C. has been confirmed by several others. Fisher reported that the thermostability varied with the strains tested, from destruction at 60°C. to only partial destruction at 100°C. for one-half hour (72).

Experiments with the common adsorbing materials were in-

conclusive (72, 87), but it appears that coagulase may be adsorbed on certain types of filters, Berkefeld "V" and "N" allowing passage of the greatest amount of coagulase (72, 87, 249, 256). Fisher called attention to the fact that an amount of calcium sufficient to clot plasma may be washed from both Seitz and Berkefeld filters (72). Thorough washing of filters with normal sterile saline obviates this factor.

The active principle of coagulase may be precipitated from a filtrate by alcohol, acetic acid, or half-saturation with ammonium sulfate, although some loss or destruction may occur (72, 256). The heat-resistance of the alcohol-precipitate is like that of the filtrate (72), while the acetic acid precipitate is less resistant to heat and to storage (256). The active principle may be dialyzed through cellophane after precipitation with alcohol, but not before (256), nor does it dialyze through collodion (87, 249).

Gratia (100) demonstrated coagulase in the filtrate of a culture that had been lysed by bacteriophage. This could not be confirmed by Pijoan (204). Fisher obtained results similar to those of Gratia with two strains of staphylococci, but with eight other strains there was either considerable loss of coagulating power or complete inhibition (72).

The antigenicity of coagulase is uncertain. The existence of an anticoagulase has been suggested, but it is largely hypothetical, and has not been conclusively demonstrated. Human or horse sera containing demonstrable antihemotoxin do not inhibit coagulase (44, 108, 147). Attempts at immunization with an active filtrate have not been successful (105, 106, 240, 256). The chief evidence suggesting the possibility of inhibition of coagulation by sera has been inferred from occasional samples of blood from cases of human infection which were slow to coagulate (108, 256). However, Sudhues and Schimrigk (240) and Cruikshank (44) were unable to demonstrate any such alteration in coagulability of plasma from cases of staphylococcal infection.

Fibrinogen solutions are clotted by coagulase, but this usually takes place more slowly and less completely than with whole plasma (72, 88, 96, 97, 256). The action of thrombin is suggested, but coagulase is unaffected by "antithrombin" such as hirudin and heparin (72, 98, 256).

FIBRINOLYSIN

The ability to liquefy fibrin, which has been widely studied in hemolytic streptococci (246), is also possessed by some pathogenic staphylococci. Important differences exist, however, between staphylococcal and streptococcal fibrinolysins. In contrast to the rapid action of the latter, which is often a matter of less than one hour, staphylococcal fibrinolysin often requires several days to bring about dissolution of a plasma clot, and many hours to liquefy a clot obtained from fibrinogen solution (73). A serologic distinction between staphylococcal and streptococcal fibrinolysins has been made (173, 194). When many hours of incubation are required to demonstrate fibrinolysis, complicating factors are introduced by the retraction of the clot from the sides of the tube, and by the tendency of fibrinogen clots to spontaneous lysis (246). Of less importance in the more rapid streptococcal fibrinolysin tests, these factors may seriously interfere with the interpretation of staphylococcal fibrinolysin tests. A slow liquefaction of plasma clot by certain bacterial contaminants (*B. subtilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Alkaligenes fecalis*, and diphtheroid bacilli) has been described by Fisher (73). Tillett (246) has suggested that this might be the result of proteolysis.

Relatively few strains of staphylococci from human pathologic sources were reported to dissolve plasma clot by Tillett and Garner (247), Fisher (73), and Neter (194). A larger proportion was found by Fisher to dissolve fibrinogen clots, and Aoi (3) reported that 88 per cent of staphylococci dissolved Congo-red-fibrin. The fibrinolytic capacity of pathogenic staphylococci is confined almost exclusively to strains from human sources. None of 24 veterinary strains studied by Madison, and Madison and Dart was active against human fibrin, and all but two of these were inactive against fibrin from a variety of animals (173, 174). These two, isolated from abscesses in horses, dissolved only horse fibrin.

Madison (173) found that 90 per cent of strains from "internal human lesions" (septicemia, osteomyelitis, empyema, cellulitis) were fibrinolytic, while 77 per cent from superficial lesions (acne, boils, nasal sinus, etc.) were inactive against human fibrin.

When tested against animal fibrins, the human strains were found to possess varying lytic capacities; of the active strains, about one-half dissolved the fibrin of only one species, and the remainder were active against two or more fibrins (173). Throughout their work Madison, and Madison and Dart used serum-free fibrinogen clot, with precautions calculated to avoid the complications imposed by this method.

Fibrinolysin has occasionally been obtained in culture filtrates, has been precipitated out with alcohol or acetone (3, 73), and subsequently concentrated (73). Madison, and Madison and Dart concentrated unfiltered broth cultures by precipitation with alcohol, in a manner previously employed by Madison for streptococcal fibrinolysin (172).

Aoi reported the production of antifibrinolysin by the intravenous injection in a rabbit of an "autolysate" or an acetone-precipitate (3). This does not appear to have been confirmed by others. Neter (194) has described inhibition of fibrinolysin by commercial staphylococcal antitoxin, and by the serum of one patient with osteomyelitis.

In common with several other bacterial species, staphylococci produce an anticoagulant, which must be distinguished from fibrinolysin (194). It inhibits coagulation of both human and animal plasma, is produced in broth containing fermentable carbohydrates, but not in plain broth, and is not antigenic. Tillett (246) has suggested that the anticoagulating activity of some bacteria, e.g., *Streptococcus viridans*, pneumococci, may be related to the pH of the medium or to products of hydrolysis of carbohydrates.

ENTEROTOXIN

In recent years outbreaks of food poisoning due to staphylococcal contamination of foods have been recognized as being clinically distinct from other types of food poisoning. Dolman (60) cites several such outbreaks involving at least 500 persons in the five years up to 1934. Since then several more outbreaks have been reported, involving about 2000 more individuals (17, 38, 39, 43, 45, 46, 47, 53, 61, 116, 131, 132, 135, 186, 187, 196,

208, 215, 227, 232, 241, 251). The geographic distribution of these outbreaks is of interest, for practically all to date have been in the United States and Canada.

A variety of foods has been incriminated in staphylococcal food poisoning. Chief among these are bakery goods (17, 38, 39, 42, 45, 53, 61, 131, 132, 186, 196, 208, 227, 260), and milk and ice cream (5, 43, 187, 215, 232, 233, 241). In addition, single outbreaks have been traced to such foods as cheese (131), meat and gravy (46, 135, 252), salads (116), oysters (175), and fish cakes (145).

The clinical symptoms of staphylococcal food poisoning include rapid onset (usually two to four hours after consumption of the contaminated food), vomiting, diarrhea, nausea, dizziness, and prostration. Recovery is usually rapid, and the individual is generally quite normal twenty-four hours later. No fatalities have been recorded. A fatal case, reported by Blackman (9), which began with the symptoms of staphylococcal food poisoning, actually involved an acute staphylococcal infection of the small intestine, and septicemia.

Study of enterotoxin has been made difficult because none of the usual laboratory animals is susceptible to oral administration of the toxin. Monkeys and human volunteers have been used, but considerable variation in susceptibility detracts from their value (47, 136, 187, 262). Furthermore, monkeys are rather expensive for many laboratories, and the use of the human subject is often inexpedient. Experiments by Borthwick (16) suggested that the apparent insusceptibility of animals might be due to the pH of the stomach contents. Using the ordinarily refractory guinea pig, Borthwick was able consistently to produce symptoms of food poisoning by adjusting the pH of the stomach contents to 7.3. Dolman, Wilson, and Cockcroft (63) were unable to reproduce these results.

A possible solution to the problem of a suitable experimental animal is suggested in the report of Dolman, Wilson, and Cockcroft (63), who demonstrated typical symptoms of vomiting, diarrhea, and weakness in kittens following the intraperitoneal injection of formalinized or heated toxic filtrates. This has

recently been confirmed by Gwatkin (112), Kupchik (160), Majors, Scherago, and Weaver (175), and by ourselves (unpublished). The intravenous injection of kittens or monkeys has recently been suggested by Davison, Dack, and Cary (51) as possessing certain advantages of sensitivity and economy of materials.

A distinguishing feature of enterotoxin is its thermostability. It is not destroyed by exposure to 100°C. for 30 minutes. Thermostability has recently been questioned by Rigdon (224), who was unable to produce symptoms in puppies or kittens following the intraperitoneal injection of a toxic preparation which had been heated at 100°C. for two hours. Unheated toxin not only caused vomiting and diarrhea, but killed the animals within four hours. It is possible that the extreme length of exposure to heat might readily destroy any toxic factors present, including enterotoxin.

Formaldehyde in the concentration used in the preparation of staphylococcal toxoid has no destructive effect on enterotoxin (63). Jordan, Dack, and Woolpert (134) reported that heating in the presence of N/100 NaOH or N/100 HCl destroys enterotoxin, and that the toxic factor is removed from acid aqueous solution by ethyl ether or chloroform. It is non-volatile and non-dialyzable. It is less readily adsorbed on filters than is exotoxin (262). Storage at low temperatures for 67 days weakens but does not destroy enterotoxin (134).

Human subjects who have repeatedly taken enterotoxin by mouth have shown only a low degree of tolerance (47). Possibly the enterotoxin was destroyed by the digestive juices, or eliminated too rapidly to stimulate antibody-formation, for immunity has been established in experimental animals. Woolpert and Dack (262) produced active, but not passive, immunity in a rhesus monkey, protecting against oral and intravenous administration of enterotoxic filtrates. Dolman, Wilson, and Cockcroft (63) reported the production of active immunity and some degree of passive immunity in kittens. Further evidence of the antigenicity of enterotoxin has recently been supplied by Davison, Dack, and Cary (51).

Reports of Woolpert and Dack (262) and of Dolman (60) appear to show that enterotoxin is a distinct entity, separable from other toxic substances of staphylococci. This is indicated by differences in thermostability and adsorptive capacity, and by serologic evidence. While it is often possible for highly potent exotoxic filtrates to be free from enterotoxin (60), it appears that enterotoxin is usually not found in the absence of such exotoxic factors as the lethal, dermonecrotic, or hemolytic toxins (60, 63, 160, 262). However, strains of proved enterotoxigenic capacity have been reported which produced no hemotoxin (53, 239, 262).

The symptoms of vomiting and diarrhea are not peculiar to the action of enterotoxin, for, as described above, a characteristic pathologic action of exotoxin in laboratory animals is diarrhea, and both vomiting and diarrhea occurred in the Bundaberg patients. Rigdon (224) noted vomiting in puppies and kittens after feeding sterile nutrient broth. The action of exotoxin may be eliminated by destruction with heat or with formalin (63, 262), or by neutralization with antitoxin (60, 262). Such a filtrate, which originally contained both lethal and enterotoxic substances, after suitable treatment produces only gastrointestinal symptoms, with recovery. The pharmacologic action of enterotoxin has not been investigated.

Identification and source of food poisoning staphylococci

In their biologic and biochemical reactions the staphylococci producing enterotoxin do not show any characteristics distinguishing them from other pathogenic staphylococci (36, 45, 131, 160, 232, 239). Agglutinin and agglutinin-absorption tests provide no basis for differentiation (239). Although the enterotoxic capacity of staphylococci may sometimes be restored by growth on starch agar (133), the medium is not differential (232).

In an attempt to identify food-poisoning staphylococci culturally, Stone (56, 235, 236) has proposed media containing gelatin in a beef-extract base, to be used for isolation and differentiation on the basis of gelatin-liquefaction by enterotoxic strains. A high degree of specificity was claimed. Toxigenicity of his

cultures was determined by feeding experiments with kittens and by epidemiologic findings. Hucker and Haynes (129) found that enterotoxigenic strains liquefied Stone's medium more readily than the usual nutrient medium. Stone's claims have not been confirmed in several recent reports (37, 63, 112, 160), an important difficulty being lack of agreement between cultural and animal tests. Grubb (111) has emphasized the importance of feeding experiments concomitant with *in vitro* tests to identify enterotoxigenic strains of staphylococci, regardless of source. The conclusions of Chapman *et al.* (36), Hucker and Haynes (129), Chinn (37), and Cogswell, Kilbourne, and Kuhns (39) lose their effect because of lack of concomitant tests. Furthermore, while between 60 and 70 per cent of strains of known food-poisoning origin have been reported to give a positive test on Stone's medium, from 27 to 70 per cent from non-food-poisoning sources likewise react positively (36, 37). The substantial proportion of staphylococci from non-food-poisoning sources which give a positive Stone reaction must be reconciled with the claims for specificity of this *in vitro* method of differentiating food-poisoning staphylococci. The liquefaction of gelatin is of course a property exhibited by many staphylococci.

The source of staphylococci responsible for food poisoning is of considerable epidemiologic interest. The importance of staphylococci from bovine mastitis has been emphasized by Crabtree and Litterer (43), Gwatkin (112), Gwatkin, Hadwen, and Le Gard (113), and Shaughnessy and Grubb (232, 233). Small local supplies of raw milk on farms have frequently been the source of outbreaks, but large urban supplies have not been implicated.

Human sources of contamination are frequently less evident. However, staphylococci of proved enterotoxigenic capacity have been isolated from human throats (239, 262) and infections (160, 239); and in outbreaks of food poisoning pathogenic staphylococci were isolated from persons handling the foodstuffs implicated (145, 187, 227, 260). The conclusion seems to be warranted that outbreaks of food poisoning may be caused directly by contamination of food by staphylococci from human sources.

This possibility has definite epidemiologic implications, and should be carefully investigated.

Dolman (60) has suggested that only a relatively few strains of staphylococci produce enterotoxin, and then only under special conditions of environment (semifluid medium and an atmosphere high in CO_2) which might rarely be met in foods. On the other hand, experiments by Dack and his associates (49, 146) have indicated that enterotoxic substances may be produced under natural environmental conditions in meat, bread, and cake.

In the control of staphylococcal food poisoning refrigeration of foods is important, but does not solve the whole problem. In controlling staphylococci in bakery goods, the principles of pasteurization have been applied by Stritar, Dack, and Jungewaelter (238) to puffs and éclairs after they have been filled with custard. The bacteria were destroyed, with no alteration in flavor or appearance of the product.

The diagnostic use of agglutinins for staphylococci in the serum of persons with food poisoning was suggested by Shaughnessy and Grubb (233). The presence of such agglutinins in many "normal" persons, however, would seem to invalidate this method, in addition to the fact that the clinical course of the poisoning is usually too short to allow antibody-formation.

DIFFERENTIATION BETWEEN PATHOGENIC AND NONPATHOGENIC STAPHYLOCOCCI

The problem of distinguishing between pathogenic and nonpathogenic staphylococci has constantly recurred in investigations on these organisms. Various criteria have been used in the past, including source, chromogenesis, and hemolysis. To these may be added the coagulase reaction and the ability to ferment mannitol, which have received fresh emphasis in recent studies. The term "pathogenic" is, of course, only relative, and has been used somewhat indiscriminately when applied to staphylococci (243).

The source of a staphylococcus may imply pathogenicity only under strictly limited conditions. That presumably nonpathologic sources may yield staphylococci of pathogenic potential-

ity is indicated by the reports of Thompson and Khorazo (243) and of Hallman (114). The former found that 21 per cent of 191 strains from normal mucous membranes fell into a pathogenic group, on the basis of precipitin tests. Hallman found that from 40 to 60 per cent of staphylococci from the normal nose were potential pathogens, as indicated by the coagulase test. Hallman's results have been corroborated by McFarlan (189).

The formation of golden pigment and the ability to hemolyze erythrocytes are frequently associated with pathogenicity of staphylococci. These criteria, particularly chromogenesis, have figured largely in many schemes of classification of these cocci. However, it can no longer be assumed that either criterion, *per se*, is indicative of the pathogenic potentiality of any given strain of staphylococcus. While it is undoubtedly true that the majority of pathogenic staphylococci are of the *S. aureus* type, it is also common experience that there is considerable variation in the degree of pigmentation. Add to this the subjective difficulty often encountered in classifying as *S. albus* or *S. aureus* the borderline strains which produce pale or creamy pigment, and the difficulty of exact correlation of chromogenesis with pathogenicity is apparent. Recent evidence has shown unmistakably that serious attention must be given to *S. albus* strains as possible pathogens (6, 27, 192). That the pigment itself (which resembles carotin) has no relation to pathogenicity or antigenicity was shown by Panton, Valentine, and Dix (198), Goadby (70), and Wright (263).

Dissociation of *S. aureus* into strains of varied pigmentation including *S. albus* strains, was described by Hoffstadt and Youmans (122), and by Pinner and Voldrich (206). The dissociants were nonpathogenic, but Pinner and Voldrich reported the restoration of the *S. albus* type to the virulent *S. aureus* form by prolonged growth in homologous serum. The possibility of restoration to virulence *in vivo* of an avirulent strain is suggested by Hoffstadt and Youmans, and by Pinner and Voldrich. This provokes interesting speculation as to the rôle of such a return to virulence in the lighting up of chronic staphylococcal infections.

Thirty years or more ago a number of investigators reported

some degree of correlation of pathogenicity and hemolysis. More recently von Daranyi (50), Pinner and Voldrich (206), and Chapman and his associates (34) reported similar correlation. This has received fresh emphasis with the repeated reports of the correlation of hemotoxin, dermonecrotoxin, and lethal toxin in filtrates of cultures grown under partial CO_2 tension. It must be remembered that such correspondence can apply only to *toxigenic* strains, and that *pathogenic* strains exist which produce no hemotoxin. The lack of correlation of hemolysis on blood agar with the test-tube titration of hemotoxin has been referred to above. In view of this, and in view of the experiences of Parker (201), Flaum and Forssman (76), and Burky (21), described above, it would appear that caution must be used in applying too generally the criterion of hemolysis to pathogenicity of staphylococci.

The value of the coagulase reaction has been established in identifying staphylococci of potential pathogenicity (42, 44, 50, 72, 96, 102, 147). Its close association with pathogenicity is evident from reports of a correlation of better than 96 per cent (33, 44, 114), with a corresponding lack of ability to coagulate plasma on the part of nonpathogenic staphylococci. This correlation, the persistence of the reaction when other *in vitro* properties are lost, and its simple technique make it a readily performed, reliable laboratory procedure of undoubted value.

The fermentation of mannitol as an aid in differentiating pathogenic strains has received repeated confirmation since its use by Gordon (95) in 1903 (32, 33, 44, 65, 112, 114, 139, 243). A high degree of correlation (usually 90 to 95 per cent) with some criterion of pathogenicity has been reported. However, an appreciable proportion of nonpathogenic staphylococci, varying from 11 to 55 per cent (33, 44, 242, 243), has been reported also to ferment mannitol, rendering this test less specific.

Chapman and his coworkers (30–35) have attempted to correlate several *in vitro* tests and to determine their significance as indicators of pathogenicity. Chief among these are pigmentation, hemolysis, and the coagulase reaction, which are interpreted in relation to each other. Good correlation of these tests

with source of the strains has been claimed. On the whole the results of Chapman and his associates serve to bear out the broad relationship between pigment, hemolysis, and pathogenicity which has been assumed by many. That exceptions occur in this general relationship is demonstrated by careful study of their tables and recorded results. The ultimate emphasis on the coagulase reaction is apparent. The limitations of source, pigmentation, and hemolysis as criteria of pathogenicity, referred to above, apply here with equal weight.

Additional *in vitro* methods described by Chapman *et al.* include the "crystal-violet reaction" (30, 31), and the use of brom-thymol-blue agar (35) and phenol-red-mannitol agar (32). Well over 90 per cent correlation of tests in these three media with the major *in vitro* tests of pigmentation, hemolysis, and the coagulase reaction was claimed by Chapman and his associates. The brom-thymol-blue and phenol-red-mannitol media were proposed for isolation. Neither is inhibitory toward other bacteria, and it would appear that the existence of an appreciable number of mannitol-fermenting nonpathogenic staphylococci would detract from the value of the phenol-red-mannitol medium for general use. It may, however, be of particular value in isolating the cocci in cases of chronic conjunctivitis (244).

In papers by Dudgeon (65) and by Winslow, Rothberg, and Parsons (261), the staphylococci are considered to be one large group which includes organisms exhibiting a varied range of biologic and pathogenic capacities. At one extreme is the deeply pigmented, hemolytic, mannitol-fermenting, precipitin-forming, pathogenic *S. aureus;* at the other, approached by the gradual loss of certain characteristics, is the white, possibly non-hemolytic, mannitol-negative, feebly pathogenic *S. albus,* with or without the capacity to form precipitins. A somewhat similar view is proposed by Chapman and his associates (33). According to them, hemolysis, pigmentation, and the coagulase reaction tend to disappear in the order listed, followed later by the other *in vitro* reactions. They have interpreted these variants, or degeneration forms, as possessing varying degrees of pathogenicity, as evidenced by their capacity to react positively to the

in vitro tests. It would appear that the evidence supplied by non-hemolytic, unquestionably pathogenic, staphylococci invalidates the application of the term "degenerate" to strains which do not possess certain *in vitro* capacities (53, 239, 262).

In summary, then, a number of *in vitro* reactions have been proposed for the identification of pathogenic staphylococci. While hemolysis and pigment-formation frequently parallel the coagulase reaction, variations occur sufficiently often to render them inadequate, *per se*, and only suggestive at best. Based upon the evidence in the literature and his own experience with all of these *in vitro* tests, it is the considered opinion of the reviewer that the coagulase reaction alone is a sufficient *in vitro* indicator of the pathogenic potentiality of staphylococci. The fermentation of mannitol supplies a valuable confirmatory test. It would seem that the use of numerous *in vitro* tests serves only to multiply confusion, particularly if one is forced by disagreeing reactions to postulate varying degrees of pathogenicity.

SEROLOGIC CLASSIFICATION OF STAPHYLOCOCCI

Agglutinins

Attempts have been made to differentiate staphylococci by serologic reactions, and to show some correlation between pathogenicity and the groups thus established. Although some of the earlier investigators, beginning with Kolle and Otto (157), reported some differentiation between staphylococci from infectious sources and saprophytic forms by means of agglutination tests, there appears to have been no recent satisfactory distinction of staphylococci by this method.

In 1922 Julianelle (138) and Hine (119), studying 25 and 81 strains, respectively, reported the use of agglutinin absorption to establish three major serologic groups, and two sub-groups of staphylococci. Julianelle reported no correlation of serologic grouping with hemolysis or pathogenicity, but he used the relatively insensitive horse erythrocytes, and recorded very few virulence tests. Hine found a general, but not exact, correspondence of the major groupings with pigmentation and fermentation of mannitol, but he recorded no attempt to demon-

strate animal pathogenicity. Unfortunately in neither of these reports is it definitely certain that reciprocal agglutinin-absorption tests were employed. While suggestive, the validity of their groupings must therefore remain in doubt, for, as pointed out by FitzGerald and Fraser (74), only reciprocal tests can provide accurate means of establishing the identity of organisms.

Seedorf (231) used agglutinin-absorption tests to confirm types previously established by means of complement fixation. Reciprocal agglutinin-absorption tests were used by Stritar and Jordan (239) in a study of 94 strains, and by Hopkins and Barrie (128) with an unstated number. The former reported results similar to those of Hine, but no close correlation of biochemical reactions or of hemolysis with grouping was obtained. Hopkins and Barrie established three groups, one containing frankly pyogenic staphylococci, but considered their results as no more than suggestive of a relationship between grouping and pathogenicity. It is probably significant that in essentially all reports on the use of these tests some strains of staphylococci could not be placed in any serologic group.

Precipitins

From several recent reports it appears that the ability of staphylococci to form precipitins may be correlated with pathogenicity. Dudgeon and Simpson (66) noted this correlation, which also extended broadly to the production of pigment and fermentation of mannitol.

Burky (24) has demonstrated a correlation of precipitinogen with pathogenicity which was found to hold for about 75 strains. He established three groups on the basis of the pathologic effect in rabbits of the intravenous injection of 10-day-old broth cultures or their filtrates. One group was definitely toxigenic, killing rabbits within two days, without abscess-formation. The second group produced no toxin, but killed rabbits in from one to 30 days with abscess-formation. The third group was nonpathogenic. Correlation of pigmentation and hemolysis with grouping was not exact. Only members of Group I stimulated the formation of precipitins, but both Groups I and II

gave precipitin reactions with culture filtrates. Members of the nonpathogenic Group III were inactive. We have been able both to confirm Burky's groupings by means of reciprocal agglutinin-absorption tests, and to correlate the grouping with pathogenicity for rabbits, the coagulase reaction, and the fermentation of mannitol (10, 13).

Julianelle and Wieghard (140–142, 259) studied the possible relation of staphylococcal polysaccharides to serologic grouping. In a study of 16 strains two types were established on the basis of precipitation of the purified carbohydrates by antibacterial sera. The pathogenic group was designated as Type A, and the nonpathogenic group as Type B. The assumption of pathogenicity, based on source of the strains, was confirmed with a few representative strains by animal inoculation by Burky's method. Chemical differences in the type carbohydrates was demonstrated by optical rotation, and by differences in the end-products resulting from hydrolysis. Neither carbohydrate alone stimulated the formation of antibodies. A correlation of types and fermentation of mannitol was later reported (139).

Julianelle's groupings have been confirmed and extended by Thompson and Khorazo (243) in a study of 286 strains, and by Cowan (42) with 157 strains. Their groups A and B correspond to the Types A and B of Julianelle and Wieghard. In both reports new groups were established which represent strains showing serologic differences from Groups A and B, and a heterogeneous group of strains which could not be allocated to specific groups by precipitin reactions. A general correlation of grouping with pathogenic potentiality is indicated by the inclusion of from 75 per cent (Cowan) to 78 per cent (Thompson and Khorazo) of strains from human infections in Group A. These strains likewise exhibited the properties generally considered as indicative of pathogenicity, such as pigmentation, hemolysis, the coagulase reaction, and fermentation of mannitol.

Cowan (42) has proposed a classification of staphylococci based on their biologic activities and precipitin reactions. The biologic classification (146 strains) represents a modification of that proposed in 1907 by Andrewes and Gordon (2), with the addition

of hemolysis. Pigmentation is not considered in Cowan's schema. Those strains causing hemolysis are included in the species *S. pyogenes*, which is further subdivided into varieties α, β, and $\alpha\ \beta$, according to whether they produce one or both of the hemotoxins described by Glenny and Stevens. *S. pyogenes* α and $\alpha\beta$ strains ferment mannitol. The nonhemolytic strains, which usually do not ferment mannitol, are classified as *S. epidermidis*. A general, but not exact, correlation of the biologic and serologic classifications is observed.

The possible influence of dissociation upon the antigenic relationships of staphylococci has been suggested by the work of Hoffstadt and her associates (120–124). *S. aureus* was dissociated into several rough and gonidial forms, differing from the original strain in biochemical and antigenic reactions, and in virulence. They showed that the smooth, rough, and gonidial strains each possessed a distinct antigenic mosaic, as demonstrated by agglutinin reactions. Specific soluble carbohydrates were produced from both the smooth and rough forms, and recently the changes in structure of the bacterial proteins during dissociation were studied.

In summary, it would appear that a broad division of staphylococci into pathogenic and nonpathogenic varieties may be made on the basis of precipitin reactions. That further subdivision might be possible is suggested by the establishment of groups showing varying degrees of serologic relationship. The necessity of including a number of strains, chiefly nonhemolytic, in one "unclassified" group indicates the heterogeneity of these organisms, which may or may not be clarified by further investigation. It may be that the presence of antigens common to the species or of antigenic mosaics which are not readily distinguishable may interfere with attempts at a finer subdivision of the species.

MODE OF INFECTION BY STAPHYLOCOCCI

As a result of the recent work on staphylococci, their toxins have received particular emphasis as important factors in the pathogenesis of staphylococcal infections. Although staphylococci have long been recognized as pyogenic organisms, it has recently been suggested that their pathogenic action is due

primarily to their toxins, rather than to their pyogenic capacity (e.g., 59, 110, 137, 214). On this basis staphylococcal toxoid and antitoxin have received wide therapeutic trial.

From experimental and clinical observations it appears quite possible that staphylococcal toxin may contribute largely to the development of lesions, but care must be used in applying the experimental findings to an interpretation of the pathogenesis of human infections; it must be remembered that rather massive doses of toxin have often been used experimentally. Although lethal toxin produces a dramatic effect in experimental animals, its rôle in human infections is probably subordinate. However, a fatal termination in acute staphylococcal infections may sometimes be quite rapid, with little evidence of invasion by the cocci, and is conceivably due to the lethal toxin. This would seem to be true of the deaths in the Bundaberg series. Although intravascular hemolysis is frequent in animals dying after the intravenous injection of exotoxin (143, 192, 193, 262), it must be remembered that human erythrocytes are relatively insusceptible *in vitro* to staphylococcal α-hemotoxin, and it appears probable that α-hemotoxin plays only a relatively minor rôle in this manifestation of the pathogenesis of human infections. The β-hemotoxin likewise does not appear to be related to human pathogenicity, nor is it of particular pathogenic importance for animals (19, 91, 182).

That staphylococcal toxin may play an important part in the development of conjunctivitis was suggested in recent work by Thygeson (244) and by Allen (1), who were able to produce conjunctivitis with certain staphylococcal filtrates. The cocci appear to develop in the conjunctival secretion or on dead cells, but do not involve the epithelium of the conjunctivae (244).

The production of lesions of the joints experimentally by the injection of toxin has been reported by Brunschwig and Jung (18) and by Rigdon (220). In considering the relation of staphylococcal toxin to the development of the conditions described, it should be remembered that similar pictures have previously been reported following the intraarticular injection of various specific and nonspecific substances (148, 149).

Possible rôles have been attributed to staphylococcal coagulase

and fibrinolysin in the establishment or dissemination of infections. This has been suggested on the basis of their demonstrated reactions *in vitro*, but lack conclusive experimental proof. Thus, it has been postulated that coagulase might be responsible for the formation of thrombi (44, 88, 106, 108). In the experimental work of Kellaway, Burnet, and Williams (143), and of Fisher (72), intravascular clotting was not obtained, nor did Fisher find any significant amount of thrombosis in 27 autopsies of cases of septicemia due to *S. aureus*. That fibrinolysin might play a part in liberating infected emboli is suggested by Fisher (73) and by Cruikshank (44).

The pathogenic importance of staphylococcal leucocidin has been repeatedly emphasized, with but little experimental evidence, except in the reports of Panton and Valentine, and Valentine (197, 248), who found a reasonably close association of leucocidin-production with certain types of human staphylococcal infection. It is to be hoped that conclusive proof of the rôle of leucocidin may soon be forthcoming.

Based upon a series of studies on staphylococcal immunity, Forssman (78–85) strongly objects to the emphasis placed upon staphylococcal toxins and antitoxins. According to him, immunity to staphylococci depends on a hitherto unknown antibody, which he describes as the "resistance factor." Following a course of intravenous injections in rabbits of a formalinized vaccine, both active and passive protection were reported against the subsequent injection of lethal doses of living staphylococci. Protective sera contained little or no antihemotoxin or antidermonecrotoxin. In contrast to the development of other antibodies, the resistance factor reached its maximum effectiveness at a considerably later period.

Kitching and Farrell (150) and Downie (64) could not corroborate Forssman's earlier work. This may be understood in the light of his subsequent work, for their protection tests were apparently done during the interval before the sera began to attain their maximum value. It does not, however, account for the failure of Flaum (75) to protect rabbits by a method almost identical with that used by Forssman. Llewellyn Smith

(170) attributed the survival of rabbits in similar experiments to the antitoxin content of their sera, but Forssman would discount this factor because of the survival of some animals whose sera contained an insignificant amount of antitoxin.

While staphylococcal toxin may figure more largely in the development of infection than has been assumed until recently, it would appear that toxin alone is not the sole etiologic factor. This is emphasized by repeated experimental demonstration that active or passive production of antitoxic immunity does not always confer complete protection against living staphylococci, although the survival time of experimental animals is often prolonged (15, 26, 40, 41, 150, 170, 191, 199, 200, 210–213, 222, 223). Likewise a state of "immunity" does not necessarily prevent the development of localized lesions (79, 84, 170). The experimental results are repeatedly confirmed by clinical observations, particularly in osteomyelitis, where they are supported by the reports of Blair and Hallman (12) and Buchman (20) that an increased antitoxin titer following toxoid therapy in chronic osteomyelitis had no effect on the clinical course of the disease.

It is important not to lose sight of the ability of staphylococci to invade tissue and to establish themselves therein. It is, of course, characteristic of most staphylococcal infections that they tend to localize in the tissue or organ affected, where, within a definitely circumscribed area the injurious action of the cocci and their products may be intense. It appears probable that in certain pathogenic staphylococci this capacity far outweighs any ability to produce toxin.

In a series of studies upon inflammation Menkin (176–180) demonstrated the effect of powerfully necrotizing irritants, among which *S. aureus* was particularly potent, in mechanically blocking off an area of injury, and preventing dissemination of the irritant. That blockage had occurred could be readily demonstrated by the inability of the lymphatics to remove trypan blue or bacteria which were injected into an area of acute inflammation. The dye or bacteria injected intravenously could likewise be fixed at a site of inflammation. These findings were

confirmed by Dennis and Berberian (54). The phenomenon suggests the action of staphylocoagulase (126), but Menkin and Walston reported that staphylocoagulase is unrelated to the substance which induces rapid obstruction of the flow of lymph (181). Menkin suggested that the active principle may be similar to staphylococcal leucocidin since filtrates causing the reaction brought about swelling and vacuolization of leucocytes.

Duran-Reynals (67, 68) has described a soluble "spreading factor" produced by staphylococci and other bacteria, whose action in enhancing infection is similar to that previously found by him in various animal tissues, particularly in testicle. The active principle is non-specific in its action, and is produced by many pathogenic staphylococci, in which it is generally correlated with chromogenesis and hemolysis.

CONCLUSION

The past several years have witnessed definite advances in knowledge of the staphylococci, particularly with regard to their soluble toxic substances. Although simplified methods of producing toxin have been developed, staphylococcal toxins have yet to reach a degree of purification where their properties and biologic action may be investigated with little interference from contaminating substances. The identity and interrelationships of the several toxic factors remain to be determined accurately. The exact relation of the toxins to the pathogenesis of staphylococcal infections demands further study. A more intelligent approach to the therapy of staphylococcal infections requires more information on these matters. Attempts at serologic classification, and a correlation of this with pathogenicity, appear to indicate some degree of separation of strains of pathogenic potentiality from the nonpathogenic varieties.

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THE EARLIER PHASES OF THE BACTERIAL CULTURE CYCLE

C.-E. A. WINSLOW

Yale School of Medicine, New Haven, Conn.

AND

HAROLD H. WALKER

University of Tennessee, Nashville

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THE BACTERIAL CULTURE CYCLE AND ITS SIGNIFICANCE

Our knowledge of this subject may be considered to have begun with recognition of the fact that bacteria transferred to a culture medium suitable for their growth exhibit a period of delayed multiplication or "lag." Specific study of the problem dates at least as far back as the observations of Müller (1895). He recognized three phases of the early bacterial culture cycle, lag, logarithmic increase and slackened growth and followed Buchner, Longard and Riedlin (1887) in computing generation times during the logarithmic phase by the formula

$$G = \frac{T \log 2}{\log b - \log a};$$

where T represents the time interval, and b and a the final and initial numbers of cells. In the same year, Ward (1895) in a brilliant and exhaustive study of *Bacillus ramosus* also determined generation times and demonstrated the three fundamental phases of slow acceleration, maximum acceleration and reduced acceleration. Furthermore, he indicated clearly the effect upon the culture cycle of temperature, of light rays from the violet end of the spectrum, of food, of oxygen, of dilution and of antiseptic substances.

The phases of the culture cycle were analyzed in a broader sense

by Rahn (1906) and Lane-Claypon (1909), who recognized four phases, lag, logarithmic increase, stationary population and decrease. McKendrick and Pai (1911) attempted to explain assumed changes in growth rate during the culture cycle as manifestations of autocatalytic reactions, the governing factors at any moment being bacterial numbers and amounts of available nutriments. Chesney (1916) made valuable contributions to our knowledge of the culture cycle, as did Buchanan (1918 and 1925) who recognized seven phases, instead of four,—initial stationary phase, lag phase, phase of logarithmic increase, phase of negative acceleration, phase of maximum stationary population, phase of accelerating mortality and phase of logarithmic mortality.

The lag period was specifically studied by Hehewerth (1901) and Whipple (1901), although the phenomenon had been noted by many earlier bacteriologists in connection with multiplication of bacteria in water samples and in relation to the "bactericidal properties of milk," (for further references, see Winslow, 1928). It was carefully analyzed by Rahn (1906), Coplans (1910) Penfold (1914), Ledingham and Penfold (1914) and Chesney (1916).

That the phenomenon of lag had a biological basis was indicated by Müller (1895) who showed that when cultures of differing ages were used for inoculation into a new medium, the generation times in the new medium differed widely. When the source culture of typhoid bacilli was $2\frac{1}{2}$ to 3 hours old, the generation time was 40 minutes in the new medium; when the same culture was $6\frac{1}{4}$ hours old the generation time was 80 to 85 minutes; when the source culture was 14 to 16 hours old, the generation time was 160 minutes. Similarly, Barber (1908) and others showed that when transfer was made from a culture in the logarithmic phase, to the same medium and under the same conditions, the new culture multiplied at once at a logarithmic rate.

It was Müller again, in 1903, who first studied the chemical activity of bacteria at various stages of the culture cycle. He did not draw any conclusions as to the ratio of end-products formed to bacterial numbers but comparison of the various tables in his paper makes it clear that the amounts of carbon dioxide and

hydrogen sulfide per cell must have been much greater in the earlier phases of the culture cycle. This phenomenon was clearly recognized at a much later date by Bayne-Jones and Rhees (1929) for heat production, by Cutler and Crump (1929) for liberation of carbon dioxide and by Stark and Stark (1929a) for acid production.

A third differential characteristic of specific phases of the culture cycle is resistance to various harmful environmental influences. Many early observations showed that very old cultures were characterized by low resistance; but this might be due merely to degenerative changes. Much more significant was the discovery by Reichenbach (1911) that young cultures in the lag and early logarithmic phase were more sensitive to heat treatment than those at the peak of their population curve and that resistance increased again in a late stage of the phase of stable maximum population. Even more striking results were reported by Schultz and Ritz (1910). These authors found that a given heat treatment (53°C. for 25 minutes) killed about 95 per cent of colon bacilli from a 20-minute culture. In slightly older cultures, resistance decreased, so that at 4 hours 100 per cent destruction occurred. Then, resistance increased again so strikingly that 7-hour to 13-hour cultures showed no reduction whatever under the same heat treatment. A markedly low resistance to harmful chemical agents was demonstrated by Sherman and Albus (1923) to be characteristic of the lag and early logarithmic phase and these authors, on the basis of their experiments, developed in a highly fruitful manner the concept of "physiological youth" as applied to the bacterial culture cycle.

A fourth characteristic of the phase of "physiological youth" had meanwhile been described by Clark and Ruehl (1919) and by Henrici (1921 to 1928). These investigators demonstrated that certain early phases of the culture cycle are characterized not only by rapid multiplication, high metabolic activity and low resistance to certain harmful environmental conditions but also by highly characteristic types of morphology, the individual cells being in general much larger than in the maturing culture. Wilson (1926) demonstrated the same phenomena by a comparison

of plate counts with measurements of the opacity of bacterial cultures.

Finally, a fifth differential characteristic was described by MacGregor (1910) and by Sherman and Albus (1923), who recorded that young cultures were more resistant than old cultures to acid agglutination. Shibley (1924) demonstrated that in the early phases, the electrophoretic charge on the cells was much less than at a later period.

The fundamental biological significance of the bacterial culture cycle was perhaps first clearly recognized by Henrici. In one of his earlier papers (Henrici, 1925a) he says:

> The cells of bacteria undergo a regular metamorphosis during the growth of a culture similar to the metamorphosis exhibited by the cells of a multicellular organism during its development, each species presenting three types of cells, a young form, an adult form and a senescent form; that these variations are dependent on the metabolic rate, as Child has found them to be in multicellular organisms, the change from one type to another occurring at the points of inflection in the growth curve. The young or embryonic type is maintained during the period of accelerating growth, the adult form appears with the phase of negative acceleration, and the senescent cells develop at the beginning of the death phase.

The same theme was developed in his later monograph (Henrici, 1928) as follows:

> The acceptance of this theory demands the acceptance of certain corollaries. If it be granted that the cells of bacteria undergo a metamorphosis of the same kind as that exhibited by multicellular organisms, then it must be granted that to this degree a population of free one-celled organisms, even though those cells have no connection other than the common nutrient fluid which bathes them, behaves like an individual. There has already been accumulated a great deal of evidence of other kinds to support the idea that there is no essential difference, that there can be drawn no hard and fast line, between populations of one-celled organisms and multicellular individuals; that a higher plant or animal is but a population of more highly differentiated cells. But there has been, in the past at least, a tendency to look upon cell differentiation in multicellular organisms as being the result of some

organizing agency peculiar to such individuals. If, however, we find in cultures of micro-organisms where no such governing agency can be supposed to exist, a differentiation of cells, even though very primitive, we are forced to conclude that such is not the case; that the high degree of organization of higher organisms is a result and not a cause of the high degree of cell differentiation.

Acceptance of the validity of this analogy between a bacterial culture cycle and a multicellular organism clarifies very greatly the long conflict between pleomorphists and monomorphists in the field of bacteriology. One group has assumed a bacterial "life cycle" governed by some inherent biological tendencies; the other group denies that a cycle of any kind exists. Both perhaps are wrong and both right. One of us (Winslow, 1935) has pointed out that variations in bacterial morphology and physiology certainly do exist but that their succession is governed by environmental and not automatic inherent factors. Furthermore, this is precisely what occurs with higher forms of life where the organism as a whole forms the environment for its individual cells.

May we not assume then, that with all living cells, the "life cycle"—so far as the individual cell is concerned—is a cycle of simple binary fission. Other phenomena involving change in cell morphology and physiology of a cyclical nature are responses to changing environmental conditions and not the result of any inherent time mechanism. If a unicellular organism shows a definite series of morphological and physiological alterations in response to certain changes in environment which are likely to occur with reasonable frequency in its natural life we may call it a "life cycle" if we wish or we may call it something else. In any case, this is the only kind of life cycle (other than binary fission) which can occur in unicellular and relatively simple multicellular forms. In this sense, the bacteria have life cycles. When we find a more complex and more regular life cycle in the higher plants and animals (relatively independent of external environment), it is because the interrelationships of the complex organism produce a cyclical change in the internal environment which is comparable with the change which takes place in a bacterial culture and which affects the individual body cell very much as the cultural environment affects the unicellular organism. (Winslow, 1935.)

The study of the bacterial life cycle is, then, the bacteriological equivalent of the study of embryology, adolescence, maturity and senescence in the higher forms. It is the purpose of the present article to review in orderly fashion some of the things we know about the earlier parts of this cycle—those included under the Buchanan phases of initial stationary, lag and logarithmic growth. No attempt will be made to cover all the literature, which would be impossible in so vast a field; but certain significant and typical data will be cited in regard to each essential point.

THE PHASE OF ADJUSTMENT

Müller, in his remarkable pioneer paper of 1895, pointed out that an inoculum from a young typhoid culture showed a much shorter generation time in a new medium than did an inoculum from an old culture. His conclusions did not deal specifically with the lag period but the length of that period obviously influenced his generation times. This is true of many data cited in the present section. Their use as illustrative of factors governing the initial stationary phase seems justified, however, since it is the length of this phase which chiefly determines early generation times. Hehewerth (1901) made similar observations. Barber (1908), working with a microscopic counting method, first showed conclusively that if transfer be made to an identical medium from a culture in the stage of logarithmic increase both stationary phase and lag disappear and multiplication continues at once in the new medium at a logarithmic rate. Lane-Claypon (1909) Penfold (1914) and Chesney (1916) demonstrated the same phenomena. Buchanan (1928) in his general review of bacterial growth curves stresses the fact that transfer from any phase of the culture cycle to an identical medium is followed by continuance of the phase which had been reached by the parent culture, cultures inoculated from either initial stationary phase, lag phase or logarithmic phase, starting in the new culture where they left off in the old one.

The term "lag phase" is commonly applied to the whole period preceding the onset of logarithmic growth; and Ledingham and

Penfold (1914) and Slator (1917) even attempted to formulate a mathematical expression to describe increase in numbers during this period. Such an analysis does not seem very profitable when one considers what radically different processes are at work. Buchanan (1918) was clearly correct in separating a primary phase of initial stationary population from that phase characterized by increase at a rate less than logarithmic. We prefer, however, to call this entire period "The Phase of Adjustment." Instead of the population being stationary during the first minutes or hours after inoculation it may often show a marked decrease. The essence of this phase of the bacterial culture cycle is the adjustment of the inoculated cells to a new medium. Its course and its length depend on the character of the inoculated cells and the nature of the inoculated medium. We agree in part with Hershey (1939) when he attributes the lag phenomenon "to initially unfavorable conditions of growth"; but we cannot agree that this phenomenon is "quite distinct from any peculiarity inherent in the cells." Whether a given condition is unfavorable or not may very clearly depend on peculiarities inherent in the cells, as shown by many observers, from Reichenbach (1911) to Sherman and Albus (1923).

We do, however, concur with Hershey in his contention that when a bacterial culture is inoculated into a *favorable* medium, an initially slow rate of increase in cell numbers cannot be interpreted as indicating "lag," in the conventional sense of low vitality. Most of the early work on this problem deals with cell numbers only; and, since we know that in the early phases of the culture cycle individual cells are of large size, a slow increase in cell numbers may not necessarily mean a slow increase in bacterial mass. Hershey and Bronfenbrenner (1938) and Hershey (1938) have shown that when source cultures of different ages are used to inoculate a highly favorable medium the rate of increase in bacterial mass and the rate of oxygen consumption per unit of mass in the secondary culture is the same for both young and old source cultures. In a more recent paper, Hershey (1939) describes even more conclusive experiments. He cultivated *Escherichia coli* in peptone beef-extract broth and for source

cultures used 3-hour and 24-hour portions of this broth culture. These portions were inoculated into the same medium and the increase in bacterial mass was measured by a photoelectric nephelometer. Simultaneously, determinations were made of bacterial nitrogen and of rate of oxygen consumption. During the first two or three hours of growth in the secondary cultures, the inocula from the young primary cultures showed a much slower rate of cell multiplication than the inocula from old primary cultures but the same rate of increase in total protoplasmic growth, as measured by either nephelometer readings, nitrogen determinations or oxygen consumption. What actually happens is that the cells from a young primary culture develop just as rapidly in total mass in the secondary culture as do the cells from the old culture; but they divide less rapidly.

Unpublished nephelometer studies made in the Department of Public Health of the Yale School of Medicine fully confirm these conclusions of Hershey, and show that the actual rate of increase of bacterial mass is nearly constant from the time growth actually begins up to the attainment of a maximum population. The "lag" in rate of cell-increase is therefore largely a result of delayed cell-division.

The "lag phase," as ordinarily defined by cell counts, may include two quite distinct phenomena, a period of adjustment, characterized by bactericidal or bacteriostatic processes and a period of normally rapid increase in mass with a low rate of cell division. In a very favorable medium, such as Hershey's, the first period disappears; in a very unfavorable medium the second period disappears. It is difficult to distinguish the two processes if cell-mass determinations are not made, and the results of Müller, Hehewerth, Barber and other investigators cited above were no doubt chiefly determined by delayed cell-division. Other data, however, clearly point to temporary bactericidal or bacteriostatic influences.

For example, injury to the inoculum before its introduction into the new medium will prolong lag, as Penfold (1914) found with prolonged chilling. Sturges (1919) noted much-delayed development of colonies on plates seeded from sewage which had

been partially disinfected by copper or sulphurous acid. Allen (1923) reported similar results (measured by generation times) following heat treatment. Recently, Hollaender and Duggar (1938) found that after exposure of *Escherichia coli* and *Serratia marcescens* to ultra-violet radiation which killed four-fifths of the cells the survivors, when inoculated into broth, showed an initial increase, followed by prolonged lag.

The phase of adjustment, as characterized by a stationary or decreasing population, was demonstrated by the early observations on bacterial changes in water samples (reviewed by Winslow 1928) where the alteration of the environment due to placing a sample in a bottle is usually sufficient to cause a temporary decrease in the mixed bacterial flora present. The fact that the bacterial count of a milk sample drawn from the udder shows a similar decrease was first pointed out by Fokker (1890); and there is an enormous literature on the "bactericidal property of milk," a problem recently discussed by Little (1937). Sherman and Curran (1924) showed that a pure culture of *Streptococcus lactis*, transferred in the stage of rapid multiplication to autoclaved milk, showed no lag but, if transferred to unautoclaved freshly drawn aseptic milk, did show a brief lag of half an hour. Sherman and Cameron (1934) found that inoculation from a peptone medium at 45° to the same medium at 10° or *vice versa* and transfer from a peptone medium to the same medium plus 5 per cent NaCl or *vice versa* all showed a considerable initial mortality in the new culture.

The results of Winslow, Walker and Sutermeister (1932) are of special interest in this connection. The strain of *Escherichia coli* studied by these investigators was cultivated in an aerated peptone medium. The addition of 0.1 M NaCl to this medium led to an initial decrease in numbers (instead of the normal lag, characteristic of the plain peptone) followed by a more rapid logarithmic rise and a higher final peak. The addition of 0.5 M NaCl caused a much greater initial decrease, followed by a delayed rise and a final peak lower than that for plain peptone. The addition of 1.25 M NaCl led to a prompt and continuing decline in numbers. Thus, we may find either a steady increase,

a steady decrease or a decrease followed by an increase, depending on the concentration of salt present.

An extremely important point affecting the adjustment of the organism to its new environment is the influence of the amount of inoculum introduced. Wildiers, as far back as 1901, showed that, even in a supposedly ideal medium, a very small inoculum of yeast failed to develop. He concluded that yeast requires for its development an unknown substance ("bios"), soluble in water, dialyzable, difficult to alter or precipitate but destroyed by incineration. Rahn (1906), working with bacteria, discussed this problem and concluded that heat-stable non-filterable substances formed by the bacteria were necessary for maximal multiplication. Penfold (1914) criticized Rahn's work (which, in fact, did not bear clearly on the lag phase on account of the long time-interval employed). Yet Penfold himself found that very small inocula did increase lag. He considered that lag was chiefly due to the lack of intermediate bodies involved in the synthesis of proteins. Chesney (1916) added the interesting observation that an inoculum of washed cells showed a greater lag than one of unwashed cells. Robertson (1923) discussed the problem from a broad biological standpoint and explained it on the ground of an autocatalytic theory of growth.

Walker (1932) demonstrated, in a particular case, that the length of the lag period can be directly controlled by concentration of carbon dioxide, which Valley and Rettger (1927) have shown to be essential to bacterial growth. Walker found that the multiplication of *Escherichia coli* in a synthetic medium could be indefinitely delayed by aeration of the culture with air free from carbon dioxide while growth could be initiated at any moment by intermitting the removal of carbon dioxide. He concludes that "the phenomenon of lag may be due largely, if not entirely, to the time it takes the culture to build up the CO_2 content of the medium or of the cells themselves to a value essential for growth." These conclusions were extended to a wide variety of bacterial species by Gladstone, Fildes and Richardson (1935).

It may be that carbon dioxide represents the "bios" of Wildiers and the "intermediate bodies" of Penfold. Such a hypothesis

might well explain the influence of the size of inoculum upon lag phenomena. We should be cautious, however, in claiming that this is necessarily the only factor at work. It is probable that it is one important factor and it certainly was the controlling one in Walker's study. Other factors may, however, be involved under other circumstances.

The nutrient value of the medium is, of course, another factor in controlling the early phases of growth. Coplans (1910), for instance, showed that lag was increased by addition of dulcitol to a peptone medium and was greater in unheated than in heated milk. Penfold and Norris (1912) showed that early generation times for *Eberthella typhosa* were increased by decreased proportions of peptone in peptone water and decreased by addition of glucose. Winslow, Walker and Sutermeister (1932) found that, when aerated with CO_2-free air, growth was easily initiated in presence of peptone while lag lasted indefinitely in a Dolloff synthetic medium.

Hydrogen-ion concentration and oxidation-reduction potential are, of course, among the most important factors governing the rate of bacterial reproduction. Cohen and Clark (1919) showed for a number of bacterial types that there is a broad zone of pH within which rates of logarithmic increase in cell numbers are fairly uniform but that on the borders of this zone, very slight changes in pH produce a marked decrease in reproductive rate. The period of "lag" is more pronounced in alkaline than in acid media. That other factors than pH may enter into the picture is indicated by the fact that the border of acid tolerance is different for acetic and for hydrochloric acid.

Fildes (1929) found that the period required for the germination of spores of *Clostridium tetani* was primarily determined by the reducing intensity of the medium. Dubos (1929) reported that growth of pneumococci, streptococci and staphylococci in meat-infusion broth was dependent on a suitable reduction potential.

Finally, the length of the stationary phase is, of course, directly related to temperature. This was shown for water samples by Whipple (1901) by Müller (1903) by Penfold (1914) and by many

others. A recent study by Anderson and Meanwell (1936) of a milk streptococcus (cultured in milk) showed a lag of half an hour at 42°, one hour at 37°, two hours at 30°, and 26°, and three hours at 20°.

Jahn (1934) has recently reviewed the problem of population growth in the Protozoa and finds essentially similar phenomena. He lists food supply, presence of waste products, pH, temperature, CO_2, oxygen tension, oxidation-reduction potential and light as the chief factors influencing development.

THE PHASE OF PHYSIOLOGICAL YOUTH

If the medium be potentially favorable, a time at last arrives when the process of adjustment between inoculum and medium is complete, either by selection of better adapted cells or by accumulation of carbon dioxide or other necessary substances. At this point the initial phase of stationary or decreasing population ceases and the phase of physiological youth begins. The change will not, of course, occur simultaneously with all the cells present in a culture. If we may visualize a given individual cell at this point it will presumably have the general characteristics of the original inoculum. We may assume that the inoculum was taken from a parent culture in the phase of maximal population, since that is the condition under which a complete life cycle is manifest. Under such conditions the culture in its early lag phase will have the following general properties.

The cells will be characterized by relatively low physiological activity (Martin, 1932; Mooney and Winslow, 1935; Huntington and Winslow, 1937); relatively small size (Bayne-Jones and Sandholzer, 1933; Clark and Ruehl, 1919; Henrici, 1928; Jensen, 1928); low multiplication rate (Rahn, 1906; Coplans, 1910; Lane-Claypon, 1909; Penfold, 1914; Ledingham and Penfold, 1914); rather high resistance to unfavorable conditions (Schultz and Ritz, 1910; Sherman and Albus, 1924; Elliker and Frazier, 1938); and relatively high electrophoretic mobility (Moyer, 1936).

As soon as the process of adjustment is completed, however, the cell in its new medium passes into a phase of physiological youth,

characterized by active metabolism and rapid increase in mass but—at first—with delayed cell-division.

The morphological and physiological manifestations of youthful activity are nearly simultaneous. Huntington and Winslow (1937), however, have shown that with *Escherichia coli*, *Salmonella pullorum* and *Salmonella gallinarum* in an aerated medium, both the initial increase and the peak of metabolic activity slightly precede the initial increase and peak in cell size. In eight sets of experiments with these three organisms in three different media, the ratio of cell size for the one-hour culture as compared with the 24-hour culture was close to unity in six instances and about 2 in the other two instances. The corresponding ratio for metabolic activity was unity in two instances and from 3 to 16 in the other six instances. Martin (1932) considers increases in cell size and metabolism to be simultaneous, but since his "simultaneous" increases represent observations at the end of a given time interval in the case of cell size and cumulative results of the entire corresponding interval in the case of metabolic activity, his results, too, fit the theory that increased metabolic activity is the first evidence of physiological youth.

Metabolic activity

The generally high metabolic activity for the early phase of the culture cycle was first indicated in the remarkable study of Müller (1903). He does not give strictly comparable figures for cell numbers and metabolic activity, but does show that carbon-dioxide production, H_2S-production and formation of products of nitrogenous decomposition all reach a high peak in the early phases of the culture cycle. If one may assume that, in these metabolic studies, the rates of multiplication were the same as those given by Müller in his other tables it is clear that metabolic activity per cell was enormous during the early lag period.

Bayne-Jones and Rhees (1929) were perhaps the first investigators to provide actual data on metabolic activity per cell per hour at different periods of the culture cycle. They studied heat production in cultures of *Escherichia coli* and *Staphylococcus*

aureus. Their most striking results were for *E. coli* in peptone broth, where the gram-calories of heat produced per cell were 60×10^{-11} at one hour, 198 at two hours, 130 at 3 hours and 75 at seven hours; but their other experiments indicated the same general relationship. With *E. coli* in plain broth, the increase was more than eightfold. Wetzel (1932) presented a formal mathematical analysis of the data of Bayne-Jones and Rhees and concluded that the formulae involved described equally well the course of heat production in a bacterial culture and in the growing human body. Schmidt and Bayne-Jones presented similar data for *E. coli* in peptone water, which yielded values of 232×10^{-11} gram-calories per cell for the first hour, 194 for the second hour, 51 for the third hour and 4 for the sixth hour.

Similar data for oxygen consumption next became available. Burk and Lineweaver (1930) showed for *Azotobacter* that rate of oxygen consumption per unit rate of increase in cell numbers was greater during the first nine hours of the culture than for the ninth to the twelfth hours. Eaton (1931), working with staphylococcus cultures, reported a higher respiration rate during the first two and a half hours. Gerard and Falk (1931) provided the first definitely quantitative data for *Sarcina lutea*. They computed a consumption of 6.5 cu.mm. of oxygen per milligram dry weight of culture for the early stages of the culture cycle as compared with 2.6 in the phase of stable maximal population. They did not, however, interpret the phenomenon as a manifestation of physiological youth but attributed it to a partial asphyxia produced in the preparation of the inoculum. Martin (1932) saw the problem in its true setting, although he did not compute rates per cell. He noted, however, that the rate of oxygen consumption reached a peak for *Escherichia coli* between 30 and 90 minutes while cell size was greatest at 60 to 120 minutes.

The fact that Hershey (1939) did not detect any change in rate of oxygen consumption at different periods of the culture cycle is not surprising since the range of ages covered in his secondary cultures was only from 1.2 to 2.3 hours. What he does show, however, is that this rate—under the conditions of his experiment—was not affected by wide variations in the age of the

primary culture from which this secondary culture was inoculated. This latter conclusion, we are quite ready to accept. As pointed out above, the physiological state of a primary culture influences the rate of growth in mass in a secondary culture chiefly through its influence on the phase of adjustment—and, in Hershey's case, the medium was so favorable that the phase of adjustment practically disappeared.

More complete information is available with regard to CO_2 production. Cutler and Crump (1929) reported that liberation of CO_2 in sands and soils was greatest per million bacteria when the numbers of bacteria were low. Walker and Winslow (1932), working with *Escherichia coli* in an aerated culture, reported 41 to 185 mg. $\times 10^{-11}$ CO_2 per cell per hour formed in the lag period against less than 2 mgm. for the close of the logarithmic phase. Walker, Winslow and Mooney (1934) studied the problem in media aerated with nitrogen instead of air and found that, under such anaerobic conditions, CO_2-production per cell per hour in a peptone medium fell from 42 mg. $\times 10^{-11}$ in the first hour to 27 in the second hour, rose to 68 in the third hour and then fell. Under anaerobic conditions in peptone-glucose the figure fell from 42 in the first to 36 in the second hour, rose to 211 in the fourth hour and then fell. The second-hour temporary decrease seems to be a peculiarity of the anaerobic state and the enormous rate recorded for peptone-glucose indicates anaerobic utilization of sugar. No increase of CO_2 production per cell was ever noted in the earlier work under aerobic conditions in sugar-containing media, as a result of the presence of the sugar, in spite of the fact that sugar was actively fermented. Walker, Winslow, Huntington and Mooney (1934) worked with *Escherichia coli* in various media and reported maximal production of CO_2 (117 to 123 mg. $\times 10^{-11}$ per cell per hour) during the second hour of the culture cycle (late lag and early logarithmic phase) falling to 16 to 22 after five hours (beyond the close of logarithmic phase). Mooney and Winslow (1935) studied *Salmonella gallinarum* and *Salmonella pullorum* as well as *E. coli*. A high peak of metabolic activity during the lag and earlier logarithmic period was always apparent. In this early phase,

79 to 145 $\times 10^{-11}$ mg. of CO_2 was formed per cell per hour as compared with 2 to 8 in the stage of maximal population. *Salmonella pullorum* in peptone-glucose had a very long lag period and the figures in table 1 give a valuable slow-motion picture of the processes involved.

Where the process is more rapid, the various stages are telescoped, so that a first-hour observation often shows a high metabolic rate; but such an experiment as that cited gives us a true picture of the processes at work.

In all the studies cited (except that of Gerard and Falk) the high metabolic rates of the early growth phases were computed

TABLE 1

AGE OF CULTURE	BACTERIA	CO_2-PRODUCTION PER CELL PER HOUR
hours	*millions per ml.*	*mg.* $\times 10^{-11}$
1	12	6
2	10	30
3	13	33
4	16	99
5	39	114
6	70	96
7	320	57
8	603	26
9	706	17
25	332	8

per cell; and these high rates of activity might be in part due to the larger size of the cells. It was clear from consideration of the magnitude of the changes involved that this could not explain the whole phenomenon; but the relationships of cell size and metabolic activity were finally clarified by Huntington and Winslow (1937). These observers combined simultaneous data on cell numbers, cell size and metabolic activity, working with *Escherichia coli*, *Salmonella gallinarum* and *Salmonella pullorum* in aerated cultures and using three different media. They computed rates of CO_2-production per cubic micron of bacterial substance and found that maximum values for the lag and early logarithmic phases ranged from 86 to 216 $\times 10^{-11}$ mg. per cubic

micron while for the phase of stationary maximum population the corresponding figures varied from 5 to 19.

A fourth measure of metabolic activity, for which similar evidence is available, is acid production. Stark and Stark (1929a) found the rate of fermentation of *Escherichia coli* to be 4.6×10^{-11} mg. per cell per hour for young cells and 0.9 for old cells. Less striking but similar relationships for fermentation by *Streptococcus lactis* have been reported by Rahn, Hegarty and Deuel (1938).

Liberation of NH_3-nitrogen is a fifth type of metabolic activity associated with physiological youth. Here, there arises the question whether lowered values for NH_3-nitrogen in later growth phases may not be due to more rapid utilization, rather than to a lessened rate of liberation of NH_3. This question cannot be categorically answered, but the close parallelism between NH_3 and CO_2 suggests that both are examples of the same phenomenon. The first suggestion of such an effect in the case of NH_3 (after the pioneer work of Müller) came from Meiklejohn (1930) who studied the relation of numbers and NH_3-production in a peptone culture of a soil bacterium. Number of cells and efficiency in NH_3-production showed an inverse relationship. Walker and Winslow (1932), in a more detailed study, determined the rate of NH_3-production for *Escherichia coli* in the lag phase in different media as 6 to 36 mg. $\times 10^{-11}$ per cell per hour while the corresponding figure for the phase of maximum population was 0.2 or less. Walker, Winslow, Huntington and Mooney (1934) reported that at $1\frac{1}{2}$ hours (late lag phase) the NH_3-nitrogen yield varied in different media from 26 to 50 mg. $\times 10^{-11}$ per cell per hour while after 5 hours (post-logarithmic phase) the values fell to 3 or less.

Hewitt (1937) reports electrode-potential curves for various organisms at various phases of the culture cycle. With hemolytic streptococci in broth the E_h begins to fall after 30 minutes and drops to a minimum in 12 hours (approximate end of the logarithmic phase). Subsequently the value rises or (with the diphtheria organism) may remain at a low level. In aerobic

glucose-broth, the streptococcus shows a sharp rise after five hours (Hewitt 1929). The phenomena involved are, however, too complex to be related with certainty to the physiological culture cycle.

Child (1929), in his striking essay on Senescence and Rejuvenescence, points out that phenomena exactly like those described above are manifest in the life history of some of the simpler invertebrates (hydroids and planaria). From the very early stages of the life of these multicellular organisms there is manifest a progressive decrease in oxygen consumption, carbon dioxide production and growth rate. Again, the analogy between a bacterial culture cycle and the life of a multicellular organism is strikingly illustrated.

Morphological changes

In parallel with the outburst of metabolic activity which characterizes the early lag period,[1] and almost—but not quite—simultaneous with it, come fundamental changes in size and other morphological characteristics of the bacterial cell.

Many of the earlier workers in bacteriology noted the presence of large cells in the initial phases of the culture cycle (see review by Ward, 1928). One of the most significant of such observations was that of Fuhrmann (1908; 1926) who observed that an organism which he called *Pseudomonas cerevisiae* exhibited a rather regular series of morphological phases in various media, beginning with small rods and passing on to large swollen cells and thread-like forms with refractive points and stainable granules. The first exhaustive study of this problem, with clear emphasis on the time-relations involved, was, however, that of Clark and Ruehl (1919).

These investigators studied 70 strains belonging to 37 species of bacteria and found that in all cases, except certain corynebacteria and the glanders organism, marked increase in size occurred

[1] These changes are commonly attributed to the *late* lag phase; but this is because under the term "lag phase" there is also included the "initial stationary phase." Using Buchanan's more penetrating analysis the characteristics discussed are associated with the very early lag phase.

in the early stages of the culture cycle. This increase generally manifested itself after 2 hours and the maximum size was as a rule noted between 4 and 6 hours. In a later review, Clark (1928) says "During the logarithmic period when maximum reproduction occurs, the cells from the young cultures of many genera of bacteria attain their maximum size, two to six times larger than the cells from the twenty-four-hour parent-cultures." During later progression, up to 18 to 24 hours, toward a stationary period the bacteria became gradually smaller in size. The large cells stain more intensely than those of normal size.

The major contribution to our knowledge of this subject came from Henrici who published a remarkable series of papers, and finally a book on the subject of Morphologic Variation and the Rate of Growth of Bacteria,[2] between 1921 and 1928. In his first paper (1921) he showed for a spore-bearing aerobe that the cells began to increase in size in the lag rather than the logarithmic phase, reached their maximum dimensions shortly after the beginning of maximum multiplication (six times those of the original inoculum), then gradually becoming shorter. *Escherichia coli* (1924a) showed maximum size at three hours (about the middle of the logarithmic phase)[3] and was down again to normal by 6 hours (end of logarithmic phase). When the cells are largest, intracellular granules disappear, the protoplasm becomes more hyaline and stains more deeply. If transferred at the moment of increasing cell size, increase proceeds; if at the moment when original size has been reached, increase begins at once; if transferred later, lag occurs (in cell size increase). The richer the medium, the longer is the period of size increase and the greater the maximum size attained (1925a). The cholera vibrio develops oval cells of less than normal curvature in the lag phase. As the logarithmic phase sets in, the cells become elongated and curved again (1925b). In Henrici's final monograph (1928) he reviews all this material with full data as to the curve of distribution of cell length and form at each stage. The phase of large

[2] Passages from this work are cited below through courtesy of Charles C. Thomas, Publisher, Springfield, Illinois.

[3] Henrici would probably have found even larger cells at a still earlier period.

cells is clearly associated with greater variability in size. The more rapid the growth, the greater is the cell size. The presence of sodium ricinoleate tends to produce very elongated cells while $CaCl_2$ (which raises surface tension) has an opposite effect. Large cells of *Escherichia coli* are more stainable and perhaps show an isoelectric point further to the acid side.

In general comment, Henrici says:

> These three correlated properties: Increased length and slenderness of the cells, indicating a greater magnitude of some axially disposed force opposing the surface tension of the medium; increased intensity of staining with basic dyes and decreased susceptibility to acid agglutination, indicating an isoelectric point of the protoplasm more on the acid side; and increased susceptibility to injurious agents, all serve to distinguish the young, actively growing cells from the resting cells, and justify our recognizing these long cells as a distinct morphologic type, as *embryonic* cells, characteristic of the growth phase of the culture.

The general significance of the phenomena involved are discussed as follows:

> It would seem from my data that the division of the growth curve into a lag phase (of accelerating growth), a logarithmic growth phase, and a resting phase, is not so significant as a division into a phase of accelerating growth and a phase of negative acceleration in growth; the so-called logarithmic growth phase when present is but a long drawn out point of inflection. For the morphologic variations which occur during the early stages of growth progress definitely to this "mid-point" (as Pearl designates it) of the growth cycle, then turn sharply in the opposite direction. The embryonic forms reach their maximum development just at the beginning of negative acceleration in growth rate, the mature forms at the end of growth.
>
> These embryonic forms will vary in their characters with different species of bacteria, but it is apparent from what has been presented here, as well as from the observations of Clark and Ruehl, that with most forms, especially the rods, they differ from the mature forms particularly in increased length and slenderness. The diphtheroid group are apparently an exception, the embryonic forms being shorter and more nearly approaching the spherical form. In all cases these embryonic forms seem to possess a higher affinity for the basic aniline

dyes. In those cases where the young cells show an increased size there is also apparent an increased variability in size. In those forms which develop intracellular granules or other structures these are lacking in the embryonic cells. In the case of the cholera vibrio, which may perhaps be taken as a type of the spiral organism, the embryonic forms are characterized particularly by straightness of their cells; they are bacillary in form.

The mature or differentiated forms, beginning to develop with negative acceleration in growth and reaching their maximum at the end of growth, are just the reverse of the embryonic forms in the characters enumerated above; . . . It is of course obvious that these cells are not differentiated in the sense that different cells show a great diversity of form and internal structure as occurs in the differentiated cells of a multicellular organism; such cannot be the case because the cells are all contained in the same environment which must be nearly uniform throughout. But the individual cells do show a differentiation in their internal structure, many forms developing within the protoplasm spores or granules of one type or another, especially volutin. Now it is just this development of internal "paraplasmatic" structures which characterizes the differentiated cells of a multicellular organism, and which are either the result or the cause of their diversified function. In this sense at least, then, there does occur differentiation in the mature cells of bacteria. (Henrici, 1928.)

It should be noted that the diphtheria bacillus seems to offer an exception to the general rules which operate with the other organisms studied. Albert (1921) recorded an early increase in size even for this organism but Clark and Ruehl (1919) found the young cells here smaller than in a later resting period. Henrici (1922), working with a chromogenic diphtheroid, confirmed Clark and Ruehl's observations but his figures (Henrici, 1928) suggest that the special tendency of this organism to form chains of streptobacilli may be the complicating factor in this case.

Meanwhile, Wilson (1926) has demonstrated increase in cell size during the earlier phases of the culture cycle by an interesting new method. He compared increasing opacity of a culture with cell-counts and found that the number of viable cells necessary to produce a given opacity was five times as great in a 26-hour culture as in a 4-hour culture of *Salmonella aertrycke*. Alper

and Sterne (1933) present similar data. The possible presence of more non-viable cells in the later phases somewhat vitiates these conclusions.

Jensen (1928) made a particularly significant study of the morphology and growth of *Escherichia coli*, with special emphasis on the history of the individual cell. His careful observations led him to the conclusion that the phase of absolute latency is one in which cell size increases without fission. He finds that, when an individual cell has reached its maximal size, multiplication at full logarithmic rate begins, so that the phase of relative latency (increase in numbers at sub-logarithmic rate) is merely a statistical characteristic of the culture as a whole. He gives us a new and very interesting type of information in regard to the proportion of individual cells in a culture which proceed to subdivide within a reasonable period of observation. At two hours (late lag or early logarithmic phase) 100 per cent of the cells exhibit prompt fission, while after four or five hours (end of logarithmic phase) the ratio falls to 10 to 54 per cent. After twelve hours, it rises once more.

The work of Clark and Ruehl and Henrici tended to show that the phase of maximum cell size and that of most rapid multiplication coincide. Jensen's studies, however, strongly suggest that increase in cell size actually precedes a rise in division rate. Thus, his colon bacilli in the first half hour were large but not yet dividing. From one to one and one-half hours (early logarithmic phase) they were large and dividing. At $3\frac{1}{2}$ hours (middle logarithmic phase) weakly-staining shadow forms began to appear. At five hours (post-logarithmic phase) the shadows had disappeared and cells were small again. These shadow forms were capable of reproduction if transferred to a new medium but apparently could not do so in the original medium.

The next important contributions to this problem came from Bayne-Jones and his associates at Rochester, using a very accurate technique based on measurement of cinematograph records. Working with *Bacillus megatherium*, Adolph and Bayne-Jones (1932), like Jensen, found that fission rates lagged behind rates of increase in total cell protoplasm. They noted

(as had Schmalhausen and Bordzilowskaja, 1926) that the rate of growth of a single cell from fission to fission is approximately constant at a given time but that the mean rate of growth in size per cell in a culture rises sharply to the end of the second hour and falls after the third hour (when an 18-hour inoculum is used). At the peak of increase in cell size the bulk of cell substance doubles every 22 minutes, and the authors compute that if such a growth rate continued for 24 hours a single filament could be produced reaching 4000 times the distance between the earth and the sun. With *Escherichia coli*, Bayne-Jones and Adolph (1932b) found that at the end of the first hour there was

TABLE 2

Time of maximum activity (hours after inoculation)

	SIZE OF CELL	RATE OF CELL MULTIPLICATION
E. coli		
Peptone	2	4
Peptone-glucose	2	3
S. gallinarum		
Peptone	3	5
Peptone-glucose	3	6
Peptone-lactose	3	7
S. pullorum		
Peptone	4	9
Peptone-glucose	4	7
Peptone-lactose	3	7

a maximum rate of growth in cell size, with no increase in numbers, while the maximum rate of reproduction occurred at the end of the second hour. Bayne-Jones and Sandholzer (1933), with *E. coli*, report that the volume of a cell of the initial inoculum was less than 1 cubic micron. At the end of the first hour this value had increased to 4 cubic micra. At the end of the logarithmic phase (160 minutes) the mean cell size was again about 1 cubic micron.

Huntington and Winslow (1937) have more recently confirmed, once more, the conclusion that increase in cell size precedes the logarithmic phase of multiplication. They studied *Escherichia coli*, *Salmonella gallinarum* and *Salmonella pullorum* in aerated

cultures using various media and found that, with eight combinations of organisms and media, the peak of reproductive activity came from one to five hours later than the peak of cell size, as indicated in table 2.

In five out of these eight instances maximum cell volume was noted during the lag phase, in the other three instances in the early logarithmic phase.

A suggestive check on these time studies is to be found in a paper by Fischer (1932) who observed the development of spreading cultures of *Escherichia coli* in soft agar. He noted in the outer zone of a spreading colony large cells (7 x 2 μ); in an inner ring, small cells (4 x 1 μ); and in the center, a region of autolysis. Here are the various time changes of Jensen, manifest at one time in those areas of a culture which are of different age.

Longworth (1938) has recently reported interesting studies of the variations in cell morphology in bacterial cultures as well as in ratios between sizes, counts and total mass as measured by a photoelectric densitometer. Hershey (1939), in the paper discussed in an earlier paragraph, expresses cell size as a ratio between nephelometric count and viable count and finds that when a 24-hour primary culture is used for inoculation the initial ratio is about 1 and increases to 3 during the first hour of the secondary culture and to 6 or 8 during the second hour. Inoculation from a 3-hour primary culture gives an initial ratio of 5 to 6, which is maintained for several hours, showing that in this respect (though not in mass-reproductive rate) the cells of a young primary culture behave differently for a time in a secondary culture from those derived from an old primary culture.

Lowered resistance to unfavorable agents

A third very important characteristic of the large actively metabolic cells of the early culture cycle is their markedly reduced resistance to various harmful chemical and physical conditions.

This phenomenon was, perhaps, first pointed out by Schultz and Ritz (1910). These investigators, using colon bacilli, exposed cultures of various ages to heat treatment at 53° for 25 minutes. In a 20-minute culture about 5 per cent survived such

treatment. In a 50-minute culture (still in the lag phase) 1 per cent survived. In 4-hour cultures (early logarithmic phase) 100 per cent were killed, and in cultures from 7 to 13 hours old (late logarithmic phase) the same treatment produced no reduction at all. These results would seem almost unbelievable if they had not been so often confirmed. Reichenbach (1911), in the very next year, reported a reduction (caused by exposure to a temperature of 47 to 51° for five minutes) of 71 to 100 per cent in 5 to 8 hour cultures and of only 3 per cent in a 28-hour culture.

The biological importance of this phenomenon was first emphasized by Sherman and Albus (1923) in their striking paper on *Physiological Youth in Bacteria.* These investigators demonstrated low resistance for *Escherichia coli* in the early culture-phases with respect to four independent conditions. They confirmed the general conclusions of Schultz and Ritz and of Reichenbach with respect to heat resistance, (although the differences were not quite so striking). They found that chilling the cultures showed a similar differential, old cultures exhibiting no reduction while young cultures did. They demonstrated the same phenomenon on transfer to a 2 per cent NaCl solution in distilled water and, finally, on exposure to 0.5 per cent phenol. In general four-hour cultures (logarithmic phase) were the ones which showed the low resistance. The results, so far as exposure to heat and NaCl was concerned, were confirmed for a *Proteus* strain. In a second communication Sherman and Albus (1924) worked out the time relations in greater detail. *E. coli* was grown at 37° in peptone-water and at intervals transferred to 5 per cent NaCl. Cells removed after one hour showed no mortality. After 90 minutes (while the parent culture was still in the lag phase) the cells showed definite susceptibility; and after 2 and $2\frac{1}{2}$ hours (when rapid increase in numbers was going on in the parent culture) mortality was greatly increased. The authors point out that this phenomenon gives clear evidence of "biologic rejuvenescence" before active reproduction begins.

The greater stainability reported by Henrici (1928) and others for the phase of increased cell size may be related to the lessened

resistance to chemical agents at this period. A later contribution in this field of resistance to chemical agents was that of Watkins and Winslow (1932) on disinfection by N/100 NaOH at 30°C. The following values for K were reported for cultures of varying age:

Age *hours*	K
8	.38
11	.22
14	.15
17	.14

Literature as to the Schultz and Ritz phenomena of lowered resistance to heat is voluminous. It was described by Ørskov (1925) for colon-typhoid strains and by Robertson (1927) for *Microbacterium lacticum*, *Sarcina lutea* and *Streptococcus thermophilus*. *S. lutea*, exposed to 71°, showed a 99 per cent reduction in a four-hour culture and no reduction in a post-logarithmic culture. *S. thermophilus*, heated at 63°, showed over 99 per cent reduction in a six-hour culture and only 55 per cent reduction in a 30-hour culture. Sherman, Stark and Stark (1929), and Stark and Stark (1929a; 1929b) made similar observations for streptococci and ropy-milk organisms. Fabian and Coulter (1930), Hammer and Hussong (1931), Heiberg (1932), Dorner and Thöni (1936) added more evidence along the same line. Hershey (1939) inoculated from 3-hour and 24-hour cultures into distilled water and found that while the young cells were completely eliminated after 15 minutes, half of the cells from the old culture were alive after one hour. It should be noted that, in many of these studies, the low resistance phase was contrasted with rather late periods of the culture cycle; so that it is not quite clear whether it is a contrast between youth and maturity or between maturity and old age of a culture with which we are dealing.

There are a few studies which appear to conflict with this considerable mass of evidence. The first of them, by Anderson and Meanwell (1936) does, indeed, seem hard to explain. These investigators worked with a thermoduric milk streptococcus cultivated in milk at various temperatures. At intervals tubes were removed and exposed to a temperature of 63° for 30 minutes.

When the parent culture was grown at 42° the lag period was very short and resistance to heat treatment decreased steadily. At 37° the lag period lasted about one hour and heat resistance rose at half an hour and then fell. In the cultures grown at 30°, 26° and 22°, respectively, lag was of course more and more prolonged but, in each case heat resistance was greatest in the early logarithmic phase. In other words, these results directly contradict all the earlier work of a score or more of different investigators. Somewhat similar results have recently been reported by Claydon (1937).

A third study which apparently conflicts with general experience appears, on closer analysis, not to do so but merely to bring out a very interesting new phenomenon with regard to the

TABLE 3

Parent culture at 28°

AGE	NUMBER OF BACTERIA PER ML.	PER CENT SURVIVAL WHEN HEATED AT 53°
hours		
0	25,000	6.4
1.5	33,800	34.0
3	40,350	1.4
6	297,000	.1
9	730,000	.02
36	3,200,000	5.8

culture cycle. Elliker and Frazier (1938 a and b) worked with *Escherichia coli* grown at 28° and 38° and then heat-shocked at 53° for 30 minutes. The results obtained are best illustrated by table 3.

The usual fall in resistance is shown for the late lag and early logarithmic phase; but the new phenomenon revealed is a temporary brief increase in resistance in the very early lag phase. More careful studies, at shorter intervals during the lag phase, showed that the initial increase in resistance lasted up to the first hour at 28° and from the twentieth to the fortieth minute at 38°. This suggests a new and exceedingly interesting characteristic of the early lag period.

The lowered resistance of the late lag and early logarithmic

phase has been demonstrated with respect to other physical influences than heat and cold by other investigators. Gates (1929) reports that a four-hour culture of *Staphylococcus aureus* is much more readily killed by a given intensity of ultra-violet light than is a 28-hour culture; and Kimball (1938) finds that budding of yeast is inhibited in a magnetic field only in the last half of the lag phase.

Bayne-Jones and Sandholzer (1933) reported that young cells are more readily attacked by bacteriophage than older ones.

A most interesting observation has recently been reported by Hegarty (1939), which may or may not be related to those characteristics of the youthful bacterial cell which make it susceptible to harmful agents in the environment. This investigator found that cells from a mature culture of *Streptococcus lactis* grown in glucose-tryptone broth can not attack galactose, lactose, sucrose or maltose until they have multiplied for some time in the presence of the respective carbohydrates. Cells from a 1- or 2-hour culture, however, attack these new sugars much more promptly. This is in the late lag and very early lag phase and the power of adaptation to the new carbohydrate, thereafter, decreases progressively. The power to utilize new carbohydrates is a favorable one while susceptibility to harmful agents is an unfavorable one; but it is conceivable that both may be related to some underlying property of ready responsiveness to the environment.

Acid agglutination and electrophoretic mobility

Finally, there is a fifth characteristic of the large and metabolically active cells of the youthful phase of the bacterial culture cycle, which may again be related to their low resistance to harmful chemical agents but is demonstrated by direct physical measurements. This is the property of low susceptibility to agglutination, coupled with low electrophoretic charge.

The first observation of this kind with which we are familiar was made by MacGregor (1910) who reported that meningococci from a two-day culture needed a concentration of 1:10 to 1:40 of normal serum to produce flocculation, while a four-day culture

sometimes flocculated with a concentration of 1:100 and at times even showed spontaneous agglutination. Gillespie (1914), working with pneumococci, found that cultures up to eighteen hours did not agglutinate in 20 hours without salt while, after twenty-four hours, they agglutinated in 6 hours. The reaction favorable to agglutination was less acid for the young cultures. Sherman and Albus (1923), working with *Escherichia coli*, reported that four-hour cultures were not agglutinated by an acidity of pH 3.0 while a twenty-four-hour culture agglutinated at a pH of 3.8.

Shibley (1924) made the first direct observation of electrophoretic charge (with pneumococci and paratyphoid organisms). In cultures from five to six hours old, the charge was low for both organisms. Between nine and ten hours, it rose and later fell slightly. Kahn and Schwarzkopf (1931) reported different phenomena for the tubercle bacillus. In four-day cultures and nine-day cultures, mobility was high, in twenty-day and five-week cultures, much lower. The tubercle bacillus is so different from ordinary bacteria and we know so little about its culture cycle that we cannot expect to interpret the data in analogous terms. There may well be a decrease in the charge on other bacteria in very old cultures. Buggs and Green (1935) indeed reported such a decrease in cultures of *Escherichia coli* and *Staphylococcus aureus* after 127 days; and these observers could find no difference between six-hour and ten-day cultures. Pedlow and Lisse (1936) also reported no change in electrophoretic mobility for *Escherichia coli* between three and twenty-eight hours.

Moyer (1936), in an exhaustive and extremely illuminating study, has given us the clearest and most complete picture of the phenomena involved. He worked with *Escherichia coli* in an aerated medium and demonstrated the following main conclusions. Rough and smooth strains exhibit distinctly different charges but, within each strain, results are highly consistent. Mobility is high at the start and falls during the first hour for the rough strain, remaining low for the second and part of the third hour and then rising again. The mobility of the smooth strain drops during the first and second hours and remains low during

the third and fourth hours. The low mobilities correspond to the lag and beginning of the logarithmic phases, and the subsequent rise in mobility comes toward the end of the logarithmic phase. Moyer made a peculiarly convincing experiment by mixing the large cells from a ninety-minute culture with the small cells from a sixteen- to twenty-four-hour culture when the differential mobility of the two morphological types was clearly manifest under the microscope. The large young cells moved at a mean rate of about 0.7μ/sec./v./cm. while the mean rate for the small old cells was about 0.9.

In a careful analysis of the causative factors involved, Moyer showed that the low mobility of the young cells was not due to irreversible changes in surface caused by buffer; nor to adsorption of dissolved metabolites; nor to adsorption of gaseous metabolites; nor to presence of flagella; but, probably, to a change in physical or chemical nature of the surface of the cells, perhaps associated with "expansion" of the surface and increased permeability. He demonstrated that heating at 56° for $\frac{3}{4}$ hour did not alter mobility of the 24-hour cells but greatly reduced the mobility of the 3-hour cells.

These results would suggest that all of the previous workers except Sherman and Albus and Pedlow and Lisse, began their observations at too late a period of the culture cycle to detect the phenomena in question. The failure of Pedlow and Lisse, Moyer ascribes to certain technical defects in their procedure, particularly to the use of distilled water as a suspending medium for the electrophoretic determinations.

THE PHASE OF LOGARITHMIC INCREASE AND THE COMPLETION OF THE NORMAL CULTURE CYCLE

It has been pointed out in earlier paragraphs that the fifth and final characteristic of "physiological youth" is a rapid rate of cell multiplication. This characteristic (which is the only one observed in ordinary bacteriological studies) is, however, initiated slightly later than the increase in metabolic activity and cell size, the lowered resistance and decreased electrophoretic charge, of the youth phase.

By the time the maximum rate of cell multiplication is reached —at about the middle of the logarithmic phase—most of the other characteristics of youth are on the decline.

Metabolic activity at this period has fallen substantially below its earlier maximum value, as shown by Bayne-Jones and Rhees (1929) and Schmidt and Bayne-Jones (1933) for heat production by *Escherichia coli*, by Eaton (1931) for the respiration of a staphylococcus, by Walker and Winslow (1932), Walker, Winslow, Huntington and Mooney (1934), Mooney and Winslow (1935), and Huntington and Winslow (1937) for production of CO_2 and NH_3 by *Escherichia* and two species of *Salmonella* and by Rahn, Hegarty and Deuel (1938) for fermentation by *Streptococcus lactis*. Schmidt and Bayne-Jones (1933), for example, reported 51 gram-calories $\times 10^{-11}$ per cell per hour produced in the phase of most rapid reproduction as compared with 232 for the lag period; and Mooney and Winslow (1935) reported 57 $\times$ 10^{-11} mg. of CO_2 per cell per hour at the period of most rapid reproduction against an earlier maximum of 114.

Cell size was noted as reduced at the middle of the logarithmic phase by Jensen (1928), Bayne-Jones and Adolph (1932b), Bayne-Jones and Sandholzer (1933) and Huntington and Winslow (1937) for *Escherichia coli*. This phenomenon did not appear in the studies of Henrici (1928) whose data for *Escherichia coli* and *Bacillus megatherium* show maximum cell size coincident with most rapid reproduction. All the more recent work, however, indicates decreased cell size in this phase. Thus, Bayne-Jones and Adolph (1932b) record a volume of 1 micron just after the point of maximum multiplication rate against 2.5 to 3.5 micra before that maximum rate. Huntington and Winslow (1937), in eight sets of experiments with three *Escherichia* and *Salmonella* species in various media, obtained a mean volume of 0.7 micron for the phase of most rapid cell-division against an earlier mean maximum of 1.1 micron.

Resistance to heat treatment was found by Robertson (1927) to be at a minimum at the height of logarithmic growth (when the "shadow forms" of Jensen, 1928, are most numerous). Elliker and Frazier (1938b) report a survival of under 3 per cent

of colon bacilli heated at 53° when the organisms were in the logarithmic phase as compared with survivals of 34 to 74 per cent for cells from earlier phases of the culture cycle.

The low electrophoretic mobility of the young cells, on the other hand, appears to persist well through the logarithmic phase (Moyer, 1936).

By the end of the logarithmic phase most of the characteristics of physiological youth have wholly disappeared.

Return to pre-lag values for heat-production at the end of the logarithmic phase are reported by Bayne-Jones and Rhees (1929), Mooney and Winslow (1935) and Huntington and Winslow (1937).

TABLE 4

Milligrams $\times 10^{-11}$ CO_2 *per cubic micron of bacterial substance per hour*

HOUR	PEPTONE	PEPTONE-GLUCOSE
First	11	82
Fourth	149	132
Tenth	17	11
Twenty-fifth	12	5

Table 4 from the data presented in the last-cited paper illustrates the general phenomenon, so far as metabolic activity is concerned.

In the peptone medium no cell multiplication had occurred during the first hour and the normal minimum metabolic activity was recorded; in peptone-glucose, however, multiplication began more rapidly and the first hour exhibits the phenomena of physiological youth. By the tenth hour, in both media, reproduction was still going on, though very slowly, and metabolic activity was nearly down to its minimum value.

Cell size was reported as down to its minimal values by the end of the logarithmic phase by Jensen (1928), Henrici (1928), Bayne-Jones and Sandholzer (1933) and Huntington and Winslow (1937). This phenomenon may be illustrated by the data (table 5) from Henrici (1928).

In each case the ten-hour culture was still increasing in numbers but was well past the height of the logarithmic phase.

The recovery at the end of logarithmic increase of normal resistance to harmful agents has been demonstrated clearly by many observers from Schultz and Ritz (1910) and Reichenbach (1911) to Elliker and Frazier (1938b). For example, Schultz and Ritz found that colon bacilli from a 7-hour culture, which had reached its peak population, were completely unaffected by heating to 53° for 25 minutes while a four-hour culture was completely sterilized by the same treatment. The recent data of Elliker and Frazier are almost as striking.

Moyer (1936) finds that at the close of the logarithmic period electrophoretic potential rises again sharply to its normal value.

After the culture cycle has passed through the phase of logarithmic increase, and the phase which Buchanan (1928) describes as that of "negative acceleration" it reaches the "maximum

TABLE 5

Size of B. megatherium, micra

HOURS	STRAIN 1	STRAIN 2	STRAIN 3
0	3.4	5.2	5.2
5	11.3	7.6	9.6
10	4.5	5.0	5.9
20	3.8	3.6	4.4

stationary phase" and then passes through the phases of "accelerated death" and "logarithmic death." With these latter phases we are not concerned in the present review. An optimum cycle, corresponding to the succession of generations in a multicellular organism, is reproduced by transferring to a new medium at the close of the period of active multiplication in the source culture. Such transfer would give us a picture in each new medium of a burst of physiological youth characterized by high metabolic activity, large cell size, low resistance to harmful physical and chemical agents, low electrophoretic mobility and rapid cell division; and, as the culture ages, all these evidences of youth gradually decrease to their initial levels. By transferring at earlier periods, the course of the life-cycle may be "short-circuited," as may be done with mammalian cells in tissue cultures.

The changes in a culture during the phases of accelerated and logarithmic death correspond to the senile changes in the multicellular organism after the normal reproductive period is passed. A comparative study of such senile changes would perhaps be timely; but they represent phenomena of a somewhat distinct type from those apparent in the early phases, which we have considered in the present review.

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BACTERIAL DISSIMILATION OF CARBOHYDRATES[1]

C. H. WERKMAN

Industrial Science Research Institute and Iowa Agricultural Experiment Station, Iowa State College, Ames, Iowa

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Professor J. B. S. Haldane introduces his Sir F. Gowland Hopkins lecture (1937) on the Biochemistry of the Individual with the statement, "The ultimate aim of biochemistry may be stated as a complete account of intermediary metabolism, that is to say, of the transformations undergone by matter in passing through organisms." In this discussion, it is our purpose to reconstruct, in the light of present knowledge, the biochemical events occurring in the living bacterial cell concerned with the dissimilation of carbohydrates. Our knowledge is far from complete; however, "Although it is dangerous to speculate too far, it is foolish not to speculate at all" (Hill).

Our remarks will be more specifically directed toward the bacterial cell although the general problem of cell physiology has received more extensive treatment with yeast and with animal cells, especially those of muscle, brain and liver. There are certain advantages in dealing with metabolic phenomena in highly specialized cells of tissue such as muscle or brain rather than the bacterial cell. In the case of muscle or brain we are dealing with cells functioning in a well-protected and constant environment with respect to pH, temperature, redox conditions, nutritional properties and other factors; whereas, a bacterial cell is virtually a street urchin of the cell world; it is found functioning at temperatures between 80°C. and freezing; at pH levels of less than 1 to above 13; under strict anaerobiosis and strong aerobiosis; with a banquet before it at one time and again subsisting on a

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diet relatively toxic. Accordingly, the bacterial cell is probably endowed with a large variety of enzymes and special mechanisms for use under widely differing conditions.

On the other hand, it is not improbable that bacteria offer marked advantages as material for study of the basic physiology of the living cell in its relationships to physical and chemical environment. More particularly those forms referred to as autotrophs show behaviors and characteristics, whose study will doubtless assist in providing an understanding of the primitive and unspecialized cell. According to generally accepted belief of biologists, life on earth is characterized by a gradual though distinct specialization of the simple forms of life to highly differentiated organisms. A spectrum may be visualized showing this transition from organisms requiring only an inorganic substrate for existence, on the one hand, to those extremely parasitic (differentiated) forms such as the viruses on the other. The degree of specialization may be indicated by the adaptability of the organism to its environment. The autotroph must have been among the early forms of life, a conception strengthened by the ease of adaptability to changing environment. In those prehistoric times bacteria must have been chemosynthetic, deriving their energy in the transfer of hydrogen to CO_2 (oligocarbophilous forms). It is likely that the use of oxygen is a more recent acquirement inasmuch as aerobes employ the anaerobic hydrogen-activating mechanism in connection with a coordinated aerobic mechanism which permits them to utilize O_2 as a hydrogen acceptor in place of some intermediately formed product as must an anaerobe. In this sense, an aerobe is an anaerobe which possesses an aerobic mechanism; therefore the anaerobic autotrophs must have preceded the aerobic autotrophs. Anaerobic dissimilation is simpler, less specialized and provides the energy requirements for organisms with low cellular differentiation. *Thiobacillus denitrificans* is an obligate anaerobic autotroph which oxidizes H_2S, S, thiosulfate or tetrathionate to sulfuric acid with the simultaneous reduction of CO_2 and NO_3. The methane bacteria are to be mentioned since some of these forms can obtain their energy chemosynthetically from the process:

$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$. The better-known aerobic chemosynthetic autotrophs, owing to their utilization of O_2, obtain relatively more energy from their substrates. Illustrative of this group are *Thiobacillus thioparus* and *T. oxidans*.

One of the early distinct steps in differentiation occurred with the origin of the photosynthetic forms. When sunlight penetrated to the earth's surface, certain bacteria accepting the possibilities offered by radiant energy used chemical energy to synthesize what was perhaps the first photosynthetic bio-energy transformer. In this case, sunlight furnishes the energy to reduce CO_2 and during the process H_2S is oxidized to sulfur or its oxides. The existence of photosynthetic bacteria had been suspected since the work of Engelmann (1883, 1888) which described bacteria showing a well defined absorption spectrum. Every effort was made to show the liberation of gaseous oxygen; failing this and recognizing the necessity for H_2S, a chemosynthetic type of metabolism was postulated and gained acceptance in the belief that the dehydrogenation of H_2S provided the energy. Such an explanation, however, did not explain the rôle of light or account for the growth of the bacteria anaerobically. It remained for van Niel (1931, 1935) to reveal the true nature of the process and to harmonize the known facts. He showed that the purple sulfur bacteria oxidize H_2S stoichiometrically to sulfate: $H_2S + 2\ CO_2 + 2\ H_2O \rightarrow 2\ CH_2O + H_2SO_4$. In the case of the green bacteria the H_2S is oxidized only so far as sulfur: $2H_2S + CO_2 \rightarrow CH_2O + 2\ S + H_2O$. Roelofsen (1935) demonstrated an endogenous liberation of CO_2 in the dark; the CO_2 was immediately assimilated in the light in the presence of H_2S. van Niel has shown that no oxygen is liberated, and that indeed, no liberation of oxygen is to be expected but rather sulfur in the case of the green forms, or its oxides in the case of the purple sulfur bacteria. Members of both the purple sulfur bacteria (*Thiorhodaceae*) and purple bacteria (*Athiorhodaceae*) can utilize molecular hydrogen and certain organic donators.

Photosynthesis appears to show an intimate relationship of chemical and solar energy-yielding processes wherein chemical energy serves to reduce the CO_2 to carbohydrate and at the same

time oxidize the chlorophyll to dehydrochlorophyll (Conant, Dietz and Kamerling, 1931). Sunlight then furnishes the energy to regenerate the chlorophyll:

$$6CO_2 + 12 \text{ chlorophyll} \xrightarrow{\text{enzyme}} 12 \text{ dehydrochlorophyll} + C_6H_{12}O_6 + 6\ H_2O$$

$$12\ H_2O + 12 \text{ dehydrochlorophyll} \xrightarrow{\text{light}} 12 \text{ chlorophyll} + 6\ O_2$$

The use of sunlight was a great step in relieving the photosynthetic bacteria of chemosynthetic chores; the necessity of providing energy was thus solved by organisms "clever" enough to use chemical energy to synthesize a converter capable of utilizing solar radiation. Since van Niel (1931) has shown that green and purple sulfur bacteria are able to reduce CO_2 by means of hydrogen from H_2S with the assistance of radiant energy, the development of unicellular chlorophyll-containing organisms would be but a step in phylogenetic development.

The gradation of bacterial metabolism may be further extended. We find the facultative heterotrophic forms which function as autotrophs but can use more complex compounds as sources of either nitrogen or carbon or both, and the facultative autotrophs preferring complex sources of nitrogen and carbon but still able to use inorganic sources. Perhaps these two groups should be recognized as one, i.e., the facultative forms, but the important point is that as the spectrum is extended from the obligate autotrophs to the obligate heterotrophs, the specialization is marked by a gradual and progressive loss of certain synthetic properties. The utilization of CO_2 by the propionic acid bacteria (Wood and Werkman, 1936, 1938) may represent a vestige of autotrophism in otherwise heterotrophic organisms.

Synthesis of chlorophyll may have been an extremely important event since it would have made free oxygen available as a hydrogen acceptor. That is, it became possible for aerobic forms of life to develop; and the use of free oxygen greatly increased the energy economy of the cell. It is likely that differentiation of the anaerobe into an oxygen-utilizing type led to the

development of series of reversible graded energy systems which released energy for use of the organism in convenient quantities resulting in a smooth, even flow. With increasing differentiation the number of oxidation-reduction systems employed by a cell became greater and successive systems involved more components to yield a smoother flow of energy.

However, differentiation and specialization among the aerobic forms also must have occurred in the course of phylogenetic development. Among the first aerobes were those which used oxygen directly as a hydrogen acceptor. This simplest of aerobic oxidation mechanisms would result in a sudden and uneconomic release of energy. It would be interesting to search for simple oxytropic systems in the primitive bacteria. If such systems do still exist, they must be rapidly disappearing with development of specialization and differentiation. Later, there developed more efficient yet more specialized types of respiration employing hydrogen carriers such as respiratory pigments and flavoproteins, and still later, types such as the cytochrome-containing forms requiring two or more carriers and oxidases.

Certain bacteria require small amounts of a considerable number of growth substances (dissimilation and assimilation factors) in their metabolism exclusive of proteins and carbohydrates. Illustrative of this group are: thiamin (as cocarboxylase) essential in pyruvate metabolism, riboflavin (yellow enzyme group), and nicotinic acid or its amide in hydrogen transportation, adenylic acid and its diphosphate and triphosphate in phosphorylation, vitamin B_6 and pantothenic acid whose functions are unknown at present.

Obviously since the autotrophic organisms do not require the addition of such factors to the medium, they must either synthesize their own or do not require them in their metabolism. Evidence supports the former assumption although instances probably may be found in which the latter assumption applies. *Aerobacter aerogenes* and *Escherichia coli* synthesize thiamin so rapidly that they cannot be depleted by growing on a thiamin-free medium, whereas the more fastidious *Propionibacterium pentosaceum* shows great stimulation on addition of thiamin to cultures

which have been grown on a medium rich in the vitamin and then transferred to a vitamin-deficient medium for not to exceed three successive transfers (Silverman and Werkman, 1938). After additional transfers *Propionibacterium pentosaceum* acquires the ability to synthesize its thiamin. The more parasitic organisms require the addition of the various factors to a synthetic medium, whereas probably many of the factors necessary for growth of the extreme parasites in artificial medium are as yet unknown. The "growth factor" requirements of organisms of this portion of the spectrum probably will be greatly extended.

Bacteria show marked ability to adjust themselves to their environment by selection and by mutation. Dissimilation of specific carbohydrates can no longer be as rigorously accepted in differentiating species or genera as in the past. Bacterial variability reminiscent of a primitive nature is of the greatest value in the study of cell genetics.

Stephenson and her coworkers have demonstrated that adaptation can occur in the parent cell, and it is probably not necessary that the cell multiply in the presence of the specific substrate in order to produce the enzyme for its breakdown. In this case, the addition of the specific substrate to non-proliferating cells is sufficient to evoke the enzyme. In other cases, the presence of the specific substrate has little to do with the appearance of the enzyme which may be effected by other factors. Thus alanine deaminase in *Escherichia coli* is not increased by the presence of alanine and may be all but eliminated by the presence of glucose (Stephenson and Gale, 1937).

It is our experience (Wiggert and Werkman, 1939) that two distinct physiological types of cells of *Propionibacterium pentosaceum* differing in sodium fluoride sensitivity and, more important, in ability to ferment phosphoglyceric acid, result from culturing in the presence and in the absence of sodium fluoride. Bacterial variation must of course be differentiated from effects due to variation in physical and chemical environment during culturing. Since mere traces of elements or compounds may be all that is required for a luxuriant growth or activity, an apparent

variation in a microorganism may result from unintentional differences in the medium.

DISSIMILATION OF CARBOHYDRATES

For present purposes, bacterial metabolism will be discussed as schematized in figure 1. The discussion will emphasize dissimilation, which may be defined as the transformation of the substrate to yield energy to the organism, as distinguished from those endothermic changes which characterize assimilation. Dis-

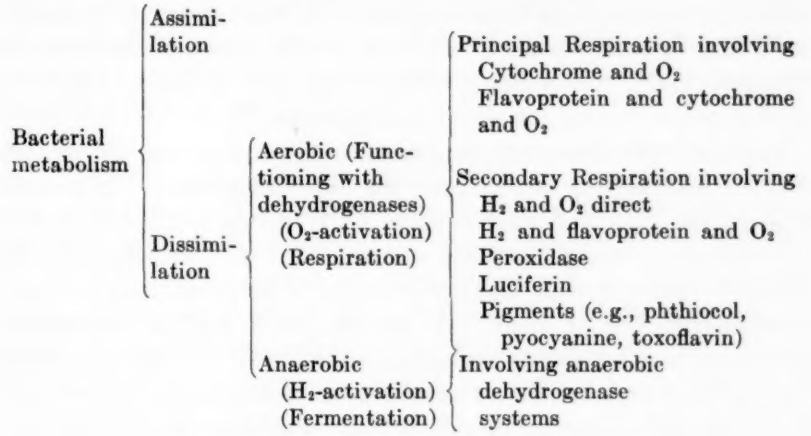


FIG. 1. SCHEMA OF BACTERIAL METABOLISM

similation may, of course, also furnish certain intermediate products necessary as building blocks in assimilation.

For convenience of discussion, we shall speak of aerobic dissimilation and anaerobic dissimilation. The former is respiration, the latter is fermentation in the Pasteurian sense. Both are truly oxidative processes.

We owe much to the genius of Pasteur who recognized that the absence of a respiratory process resulted in "la fermentation, c'est la vie sans air." It was Pasteur who recognized the *physiological equivalence* of fermentation and respiration.

Pasteur (1876) in giving a broad interpretation of what is now known as the Pasteur effect stated (free translation): "Fermenta-

tion is a very general phenomenon. It is life without air, life without free oxygen, or in more general terms, it is the result of a chemical process on a fermentable substance, i.e., capable of producing heat by decomposition."

"Fermentation—a chemical process, connected with the vegetative life of cells—takes place at a moment when these cells, ceasing to have the ability of freely consuming their substrate by respiratory processes—that is, by the absorption of free oxygen —continue to live by utilizing oxygenated substances like sugar. This characteristic (of fermentation) is always ready to manifest itself and in reality does so as soon as life ceases to perform its functions under the influence of free oxygen or without a quantity of that gas sufficient for all acts of nutrition."

Pasteur, with keen insight, has thus given us a concise picture and it is convenient to recognize his differentiation. His general concept has required modification only in one respect in which subsequent investigation has shown lack of breadth, i.e., his insistent demand that the phenomena of fermentation are correlative with life or vital activity (cf. Burk, 1937). Buchner's preparation of an active "press-juice" in 1897 did much to push Pasteur's views into the background for two decades. However, in this respect it should be noted that to date no active preparation, free of cells or cell fragments, of the respiratory enzymes has as yet been obtained. At any rate, the wisdom of Pasteur did not prevent an undue emphasis being placed on the rôle of oxygen in biological oxidation after the discovery of oxygen by Priestley and the enunciations of Lavoisier.

The terms respiration and fermentation have been variously defined and used, even by the same author. It is not so important whether one or another term is used, as it is that the term employed be adequately defined in the light of our present knowledge. Differentiation of the two processes is solely a matter of convenience and no fundamental difference is implied. The intimate relationship of fermentation and respiration is revealed in the Pasteur reaction, a phenomenon which has not been adequately elucidated but does show the quantitative inter-

dependence of the two processes. The linkage between the two reactions has never been discovered although it is generally accepted that aerobic dissimilation is always associated with fermentation, i.e., aerobic carbohydrate dissimilation is always preceded by anaerobic phases (phosphorylation and splitting), in spite of the discovery of enzymes oxidizing glucose directly, or that monoiodoacetic acid at certain concentrations inhibits fermentation and not respiration.

The allocations under Principal and Secondary respiration (fig. 1) are largely speculative and are based on results obtained with cells other than bacteria. They are given here for completeness. Future investigation will show the proper allocations and provide details of mechanism.

Biological oxidation

It is now generally accepted that biological oxidation-reduction manifests itself as a transfer of hydrogen (electrons) from donator to an acceptor, the transfer yielding energy to the organism. Clark (1923) has adequately defined biological oxidation as "the withdrawal of electrons from a substance with or without the addition of oxygen or elements analogous to oxygen; or as the withdrawal of electrons with or without the withdrawal of hydrogen or elements analogous to hydrogen." The rôle of oxygen is that of a hydrogen acceptor. Under anaerobic conditions the O_2 is replaced by some other suitable H_2-acceptor. Thunberg (1917, 1918, 1920) and Wieland (1912, 1913, 1922a, 1922b, 1925) have provided us with the basic concepts of our present knowledge of biological oxidation-reduction.

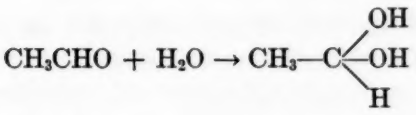
The transfer of hydrogen is activated by specific enzymes known as dehydrogenases. Wieland's original concept did not provide for the activation of the H_2-acceptor but only of the donator. It is apparent that certain of the acceptors do not require activation; e.g., methylene blue. On the other hand, probably most naturally occurring acceptors do require activation. The term "activation" has not been defined; nor will any rigid definition be attempted. For the time being, we must sense the mean-

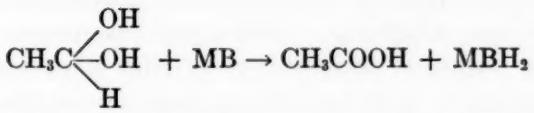
ing. Suffice it to illustrate the point: If succinic acid and methylene blue coexist in solution, no detectable chemical change occurs even after long time, i.e., the substrate is stable even in the presence of oxygen; however, on addition of an active suspension of washed cells of *Escherichia coli*, the methylene blue accepts hydrogen to become reduced methylene blue and succinic acid is dehydrogenated to form fumaric acid. In this case the succinic acid molecule has been activated to donate hydrogen to methylene blue. The trigger-like activation has initiated the the transfer of hydrogen. The term activation is used to express a general concept, i.e., that certain metabolites such as glucose, succinic acid, lactic acid, polyalcohols, etc., are so changed under the influence of certain specific cellular agents that hydrogen atoms may become transferred to reducible substances (H_2-acceptors), a transfer which otherwise would not occur (Thunberg, 1937). The phenomenon requires a designation, and the term activation is as convenient as any and does not imply the nature of the mechanism responsible (cf. Michaelis, 1933). Not only is activation necessary but the donator and acceptor must be suitable, i.e., the transfer of hydrogen must result in a decrease in the free energy of the system. It should be emphasized that the transfer of hydrogen anaerobically is as truly an oxidation as its transfer aerobically where O_2 is the acceptor. It is now becoming clear that this transfer occurs in orderly fashion and has a complex mechanism. It may occur between molecules or within the same molecule and may be symbolized thus:

$$AH_2 + B \xrightarrow{\text{dehydrogenase}} A + BH_2 \text{ (or } ABH_2)$$

$$ABH_2 \xrightarrow{\text{dehydrogenase}} AH_2B \text{(or } AH_2 + B)$$

Wieland (1925) provided support for his theory by demonstrating the oxidation of acetaldehyde to acetic acid by *Acetobacter* in the absence of free oxygen with methylene blue as the hydrogen acceptor. In this case, the aldehyde is hydrated and two atoms of hydrogen are activated by a dehydrogenase and transferred to the methylene blue.





Dehydrogenases. Ehrlich as early as 1885 injected methylene blue into animal tissues and found that most of them reduced the dye. Schardinger in 1902 discovered an enzyme in milk which reduced methylene blue in the presence of an aldehyde. It was not until a decade later that the significance of the action of such enzymes was realized and dehydrogenases were recognized as enzymes activating the release of hydrogen from the molecule of the donator, the hydrogen being transferred to an acceptor. Thunberg's methylene blue technique (cf. Ahlgren, 1936) is generally used to demonstrate the presence of a dehydrogenase. Dehydrogenases may function in the transfer of hydrogen from the donator directly to oxygen as the acceptor. Such dehydrogenases are called oxytropic dehydrogenases and apparently form H_2O_2. It is difficult to differentiate the oxytropic dehydrogenases and the oxidases. Perhaps no harm will result if we think of the dehydrogenases as activating the release of hydrogen, the oxidases as activating the oxygen as an acceptor.

Oxytropic dehydrogenases function with suitable dyes as acceptors in the absence of O_2 and may, or may not, be cyanide-sensitive. They do not require coenzymes or the cytochrome-oxidase system, and may be considered relatively simple systems in that the activated hydrogen passes directly from the donator to O_2 without recourse to carrier systems.

The anaerobic dehydrogenases are those capable of activating the release of hydrogen in a system in which the immediate acceptor is not molecular oxygen. Certain of the anaerobic dehydrogenases function through coenzymes which may in turn require a dehydrogenase, e.g., diaphorase, to activate the release of hydrogen from the reduced coenzyme. The route of hydrogen

transfer is generally devious and may not be a single or fixed path; it is not unlikely that any naturally occurring reversibly oxidizable and reducible substance of suitable potential may function as a transporter of hydrogen. Among those occurring in bacteria are riboflavin, cytochrome, coenzyme I and II, pyocyanine, phthiocol and toxoflavin. For a summary of dehydrogenase systems and bacterial fermentations the reviews of Kluyver (1935), Harrison (1935) and Potter (1939) may be consulted.

Coenzymes. In addition to the hydrogen donator, the hydrogen acceptor, necessary dehydrogenases and supplementary factors such as water, buffers and inorganic ions, many biological reactions require the presence of coenzymes. A coenzyme may be defined as a dialyzable, thermostable substance necessary in addition to the enzyme and substrate to initiate a reaction. A coenzyme is usually an organic compound although the term was first used by Bertrand (1897) to characterize inorganic ions (Ca and Mn) which activated plant enzymes. The present usage dates from the work of Harden and Young (1905, 1906) who found the thermostable, dialyzable fraction of yeast-juice necessary to initiate fermentation in the residue. Considerable confusion existed regarding the nature of yeast coenzyme (cozymase). In fact any dialyzable, thermostable substance stimulating the action of yeast press-juice was considered a coenzyme; i.e., Mg, K, PO_4, hydrogen acceptor (necessary to initiate certain reactions which continue by virtue of acceptors formed subsequently), anti-protease and Euler's principle. Owing to the work of Euler (1936) and Warburg in recent years, remarkable progress has been made in elucidating the function of coenzymes. Cozymase I of Euler and cozymase II of Warburg and Christian, both of which are adenylic acid nucleotides of nicotinic acid amide, are important H_2-carriers, capable of passing hydrogen to flavoprotein. Coenzyme II acts with hexosemonophosphate dehydrogenase; coenzyme I acts with hexosediphosphate, lactic acid (muscle), alcohol and malic acid dehydrogenases. Recently Euler and Adler (1938) have shown the biological inter-conversion of the two coenzymes.

A coenzyme may function in one or more of several ways; i.e., as a H_2-carrier, phosphate-carrier, oxygen-carrier or in ways not now clear. Euler and Myrbäck (1923) proposed the term cozymase for the coenzyme of alcoholic fermentation, whereas the enzyme free from coenzyme has been termed apozymase (Neuberg and Euler, 1931). The present tendency is to use the term coenzyme in referring to thermostable, dialyzable organic substances; e.g., Harden and Young's coenzyme (cozymase, codehydrase, coenzyme I, diphosphopyridine nucleotide), coenzyme II (triphosphopyridine nucleotide), cocarboxylase (thiamin pyrophosphate), and adenylic acid.

Although coenzymes were formerly looked upon as separate entities which accelerated the reactions brought about by the enzymes proper, our point of view is changing. The relationship may involve a union of coenzyme and apoenzyme to form the enzyme (holoenzyme); the coenzyme being a prosthetic group. On the other hand, the coenzyme may constitute a relatively separate entity. In view of recent results, both concepts or a modification may apply. It is probable that in certain cases the coenzyme is very easily dissociated, whereas with other enzymes dissociation does not occur with our present methods. The latter would be represented by those enzymes now considered as not requiring coenzymes. Theorell (1935a) concluded that the dehydrogenase combined with the substrate but not with coenzyme (coenzyme II) although the latter did combine with the substrate-enzyme complex. On the contrary Warburg (1928) insists upon the union of enzyme and coenzyme. Theorell (1935b) split the riboflavin-protein into a protein and a prosthetic group by dialysis. The protein could be combined with flavin phosphate prepared synthetically to form an active enzyme. In a number of cases the protein has been combined with a prosthetic group to yield an active enzyme. It seems that there is ample evidence that it is a question of ease of separation of the holoenzyme (proteid of Warburg) into coenzyme and apoenzyme. As new methods of separation are developed it will be found probably, that enzymes now considered to require no coenzyme will be dissociated into coenzyme and protein carrier.

There is evidence that the pyridine nucleotides (coenzymes I and II) also combine with specific proteins to form active complexes but that the latter are easily dissociated, more so than the flavin proteins.

A cell provided with suitable dehydrogenases with necessary coenzymes, H_2-donators and H_2-acceptors is still not equipped for dissimilation. The evidence seems clear that phosphorylation plays an essential rôle in the dissimilation of glucose by the normal cell and that phosphate-carrying coenzymes are necessary. Although it was formerly believed that the rôle of phosphoryla-

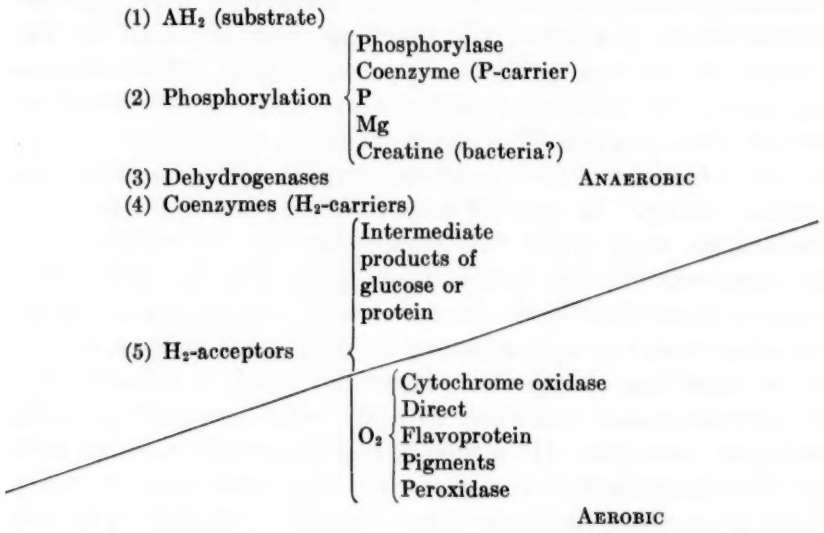


FIG. 2. COMPONENTS OF AEROBIC AND ANAEROBIC DISSIMILATION

tion was limited to the early stages of glycolysis, it is now certain that it is essential in all but the last steps following pyruvic acid. It is possible that bacteria and fungi possess in addition, a mechanism to utilize carbohydrates not involving phosphorylation (cf. Nord, Dammann and Hofstetter, 1936). Wiggert and Werkman (1938) have recently shown the association of phosphorylation of glucose and glucolysis in the living bacterial cell. Phosphorylation of glucose by tissue and yeast cells is generally accepted (Macfarlane, 1936).

A cell may be able to live an anaerobic existence from the point of view discussed; however, it may have learned also to use molecular oxygen as a hydrogen acceptor.

In figure 2 are illustrated the various types of factors necessary in anaerobic and aerobic dissimilation. The fact must be kept in mind that aerobic dissimilation is conditioned on the presence of suitable dehydrogenase systems.

Aerobic dissimilation is more economical than anaerobic dissimilation in the sense that the molal energy of glucose dissimilation by O_2 to CO_2 and H_2O is approximately a dozen times greater than the anaerobic dissimilation leading only to lactic acid as the final product.

The lactic acid fermentation may serve as an example of anaerobic behavior. We observe that much of the energy represented by the sugar remains locked within the two molecules of lactic acid (cf. Burk, 1937). This process is uneconomical. Now let us assume that the dissimilation occurs in the presence of oxygen as a hydrogen acceptor, and that it results in the formation of CO_2 and H_2O.

Anaerobic Dissimilation Involving Lactic Acid

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH$$
$$-\Delta F = 54{,}740 \text{ calories}$$

Aerobic Dissimilation to Carbon Dioxide and Water

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH$$
$$2\ CH_3CHOHCOOH + \tfrac{1}{2}\ O_2 \rightarrow CH_3COCOOH + H_2O$$
$$CH_3COCOOH + \tfrac{1}{2}\ O_2 \rightarrow CH_3COOH + CO_2$$
$$CH_3COOH + 2\ O_2 \rightarrow 2\ CO_2 + 2\ H_2O$$
$$-\Delta F = 664{,}000 \text{ calories}$$

It should be recalled that the energy released by dissimilation remains constant, so long as the final products remain the same, i.e., the available energy is independent of the path of the intermediary breakdown.

Mechanism of anaerobic dissimilation (fermentation)

It is convenient to discuss first anaerobic dissimilation and secondly aerobic dissimilation inasmuch as the aerobes appear to have developed from the anaerobes.

Bacterial dissimilation generally involves transformations in the glucose molecule or a polymer as a source of energy. For this reason glucose will serve as the substrate in the discussion of anaerobic dissimilation, and certain analogies will be drawn with schemes of metabolism which have been proposed for muscle and yeast in particular. Recent progress in our knowledge of the biochemistry of muscle metabolism has led to marked changes in our views regarding the dissimilation of carbohydrates by microorganisms, especially the heterotrophic forms.

Our knowledge of the intermediary metabolism of the autotrophic bacteria is quite fragmentary. The metabolism of the heterotrophic and more highly specialized forms bears at least in part, a similarity to that of animal cells with regard to enzymes, coenzymes, carriers and other agents. We have little information regarding the autotrophic intermediary mechanism. Our discussion then is concerned more specifically with bacteria belonging to the heterotrophic *Eubacteriales.*

Numerous theories have been proposed to account for cellular dissimilation; it has been well established that the living cell must have energy to carry on its metabolism, grow and reproduce; this energy is furnished stored in the food molecule which must be rearranged to provide available energy to the cell. This is, of course, excluding the photosynthetic bacteria which utilize the energy of the sun, as do typical chlorophyll-containing plants.

The Embden-Meyerhof-Parnas theory. The work of Embden, Deuticke and Kraft (1933) dealt with muscle metabolism. Meyerhof greatly expanded the work with muscle and extended Embden's theory to yeast. The investigations of these workers in the field of muscle and yeast metabolism have proved of inestimable value in the field of bacterial metabolism. Werkman and coworkers have presented experimental evidence that the Embden-Meyerhof-Parnas theory finds application to bacteria by their isolation of the key intermediate of that scheme (phosphoglyceric acid) from a wide variety of bacterial fermentations. Phosphoglyceric acid was first isolated in the case of bacteria from *Citrobacter freundii* (Werkman, Zoellner, Gilman and Reynolds, 1936) and later from *Escherichia, Aerobacter, Propioni-*

bacterium (Stone and Werkman, 1936a, 1936b), and organisms of a relatively large number of genera: *Bacillus, Azotobacter, Serratia, Lactobacillus, Streptococcus* and *Staphylococcus* (Stone and Werkman, 1937; Werkman, Stone and Wood, 1937). Recently Endo (1938) in Meyerhof's laboratory has confirmed and extended the work in the case of *E. coli.*

Much of the work has been carried out on cell-free juices of yeast and muscle. Satisfactory bacterial juices are difficult to prepare. Booth and Green (1938) have prepared an active juice in a roller grinder and Wiggert, Silverman, Utter and Werkman (1939) have obtained an active preparation by grinding the cells with powdered glass and centrifuging the resulting juice in a Beams ultracentrifuge. Such an active juice can be dialyzed and essential coenzymes thus removed, studied and identified. In addition to dialysis, specific inhibitors can be used to suppress certain reactions leaving others active; also, certain agents may be used to fix intermediate products for identification. Finally one arrives at results from which a general scheme of fermentation can be synthesized. Silverman and Werkman (unpublished) have prepared a cell-free juice from *Aerobacter aerogenes* capable of making the following conversion: $2\ CH_3COCOOH \rightarrow 2\ CO_2 + CH_3COCHOHCH_3$. The simple system may provide a method of attack leading to the elucidation of the synthesis of 4-carbon compounds from 3-carbon substrates. The use of dialyzed cell-free juices should permit us to reconstruct the biochemical events occurring in bacterial dissimilation. This method of attack has proved profitable in studies on animal and yeast metabolism; it should prove equally valuable in studies on bacteria (cf. Parnas, 1938a).

The scheme of Embden, Meyerhof and Parnas is given in figure 3 for reference. The reactions consist of oxidations, hydrolyses and phosphorylations.

It is generally accepted that cellular utilization of glucose involves its phosphorylation with a subsequent split into phosphorylated trioses. Hevesy (1938) and Parnas (1938b) have conducted experiments with tissues, eggs, milk and yeast fermentations with synthetic radioactive adenylic acid, in which it

was possible to trace the radioactive phosphorus. After a period of incubation, a considerable portion of the active phosphorus was found in the sugar-phosphoric acid ester fraction. Evidence indicates that this general scheme of events occurs with the bacterial cell as well as with muscle and yeast. The evidence is clear that phosphorylated hexoses occur in bacterial metabolism (Virtanen, 1924, 1925; Virtanen and Karström, 1931). Adenylic

2 glucose + adenylpyrophosphate $\xrightarrow{\text{phosphorylase}}$ 2 hexosemonophosphate + adenylic acid

2 hexosemonophosphate + adenylpyrophosphate $\longrightarrow$ 2 hexosediphosphate + adenylic acid

Hexosediphosphate $\underset{\longleftarrow}{\overset{\text{aldolase}}{\longrightarrow}}$ glyceraldehydephosphate $\rightleftharpoons$ dihydroxyacetonephosphate

$\downarrow\uparrow$ + H_2

α-glycerophosphate

$\downarrow$

glycerol + H_3PO_4

Glyceraldehydephosphate $\longrightarrow$ 3-phosphoglyceric acid $\rightleftharpoons$ 2-phosphoglyceric acid

2-phosphoglycerate $\underset{\longleftarrow}{\overset{\text{enolase}}{\longrightarrow}}$ phosphopyruvate + H_2O

2 phosphopyruvate + adenylic acid $\xrightarrow{\text{phosphorylase}}$ 2 pyruvic acid + adenylpyrophosphate

Adenylpyrophosphate + 2 creatine $\underset{\longleftarrow}{\overset{\text{phosphorylase}}{\longrightarrow}}$ adenylic acid + 2 creatine phosphate

Adenylpyrophosphate $\longrightarrow$ adenylic acid + 2 H_3PO_4

Triosephosphate + CH_3CHO $\xrightarrow{\text{mutase}}$ C_2H_5OH + phosphoglycerate

Triosephosphate + pyruvate $\longrightarrow$ lactate + phosphoglycerate

Pyruvic acid $\xrightarrow{\text{carboxylase}}$ CH_3CHO + CO_2

FIG. 3. THE EMBDEN-MEYERHOF-PARNAS SCHEME

acid and its allied phosphates act as coenzymes in the transfer of phosphate from phosphopyruvic acid to glucose. Among these adenosine nucleotides are: adenylic acid, adenosine diphosphoric and triphosphoric acids, diadenosine pentaphosphoric acid, diadenosine tetraphosphoric acid and the pyrophosphate (Kiessling and Meyerhof, 1938); all apparently function as phosphate carriers in cellular glycolysis although the active participation of all in bacterial metabolism has not been established.

Phosphorylation of glucose leads to the formation of hexose-6-phosphate which is an equilibrium mixture: glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate. There is as yet no evidence for the formation of the Cori ester (hexose-1-phosphate) (Cori and Cori, 1936, 1937) by bacteria. This ester is formed from the polysaccharides and rapidly converted into the 6-ester. The 6-monophosphate is converted into the Harden-Young ester (fructose-1, 6-diphosphate). Phosphorylation of the hexose leads by an intramolecular rearrangement to an "active" form of the sugar which is indicated to have a furanoid structure by the fact that the cardinal intermediate hexose (fructose-1, 6-diphosphate) is a furanoid. The diphosphate is the precursor of all triosephosphate which reacts with cozymase (coenzyme I). This reduction proceeds slowly (Meyerhof, 1938) unless a phosphate acceptor is present.

In most tissues the hexosediphosphate is thus converted into a complex equilibrium mixture:

$$\text{hexosediphosphate} \xrightleftharpoons{\text{aldolase}} \text{glyceric aldehyde phosphate} \rightleftharpoons \text{dihydroxyacetone phosphate}$$

The glyceric aldehyde phosphate is oxidized in a coupled reaction to 3-phosphoglyceric acid $\rightleftharpoons$ 2-phosphoglyceric acid. Initially α-glycerophosphate is formed by a reduction of a second molecule of triosephosphate. This type of reaction is called dismutation by Neuberg or more generally a Cannizzaro reaction. Subsequently the molecule reduced is not triose but a H_2-acceptor which forms later in the scheme (e.g., acetaldehyde, pyruvic acid). Enolase changes the phosphoglyceric acid into phosphopyruvic acid which is irreversibly dephosphorylated (Embden, Deuticke and Kraft, 1933) by the adenylic acid system, which may be regarded as a dissociable, active group of the enzyme, phosphorylase. The phosphate is carried by the adenylic acid (adenosinmonophosphate) as adenosindiphosphate or adenosintriphosphate, both discovered by Lohmann, to glucose which forms initially hexose-monophosphate and then the diphosphate (Harden and Young ester).

Needham and van Heyningen (1935) have reconstructed the

steps between phosphopyruvate and adenylic acid with the formation of adenylpyrophosphate and pyruvate and have shown that dephosphorylation of the phosphopyruvate is exothermic. Nature makes considerable use of phosphate transfer in connecting assimilation and dissimilation. Another reaction in which energy and phosphate are passed between molecules is that occurring in muscle extracts:

$$\text{Adenylpyrophosphate} + 2 \text{ creatine} \rightleftharpoons \text{Adenylic acid} + 2 \text{ creatinephosphate}$$

The heat of hydrolysis of adenylpyrophosphate is approximately equal to that required in the synthesis of creatinephosphate.

Creatinephosphate appears to serve as a reservoir for phosphate. It does not occur in yeast, however, and its rôle in bacterial metabolism is uncertain.

It is to be noted that the phosphate appears to work in a closed system although it has been demonstrated with muscle (Parnas and Ostern, 1936; Meyerhof, 1937), yeast (Macfarlane, 1936) and bacteria (Wiggert and Werkman, 1938) that inorganic phosphate is taken up and later released from organic combination. Needham and Pillai (1937) and Meyerhof (1937) have shown the change,

$$\text{Adenylic acid} + H_3PO_4 \rightarrow \text{adenylpyrophosphate}$$

to take place by the energy liberated from the following reaction:

$$\text{Pyruvic acid} + \text{triosephosphate} \rightarrow \text{lactic acid} + \text{phosphoglyceric acid} + 8{,}000 \text{ (approx.) cal.}$$

Pyruvic acid appears to be a general intermediary in bacterial dissimilation of glucose. It has been isolated in this laboratory in the case of a relatively large number of genera [*Propionibacterium* (Wood and Werkman, 1934), *Escherichia*, *Citrobacter*, *Aerobacter* (Reynolds, 1935), *Lactobacillus*, *Clostridium*, *Bacillus*, *Azotobacter*, among others (unpublished data)].

In the present state of our knowledge it appears that the Embden-Meyerhof-Parnas scheme of glycolysis finds application in the anaerobic dissimilation of bacteria. However, from experiments with NaF (Werkman, Stone and Wood, 1937; Wiggert

and Werkman, 1939) it is shown that the Embden-Meyerhof-Parnas scheme may not exclusively function in the dissimilation of glucose by *Propionibacterium pentosaceum* but that the organisms may possess in addition some other path of dissimilation. However, there are several facts which support the essential rôle of phosphoglyceric acid as a normal intermediary. First, it is readily isolated; secondly, it is dissimilated to normal final products (cf. Tikka, 1935). A third point is the behavior of other phosphate esters, i.e., phosphoglyceric acid can be isolated from the dissimilation of hexosediphosphate and hence the propionic acid bacteria must possess the enzyme systems requisite for glycolysis by the Embden-Meyerhof-Parnas scheme.

Further investigations may again reveal the greater versatility of bacteria as compared with the more differentiated cells of muscle and brain.

Dissimilation of pyruvic acid. Pyruvic acid may be looked upon as a cardinal intermediary in cellular metabolism. It has been shown to occur in the dissimilation of glucose by muscle, brain, kidney, yeast, fungi and bacteria. From it originate many products of cellular dissimilation such as, acetic, butyric, succinic and fumaric acids, ethyl alcohol, glycerol, acetylmethylcarbinol, 2,3-butylene glycol, acetone, isopropyl alcohol, carbon dioxide, and hydrogen. Pyruvic acid may also serve in the formation of amino acids. In muscle glycolysis the pyruvic acid is reduced to form lactic acid; with yeast it is first decarboxylated to acetaldehyde and CO_2, the former is then normally reduced to ethyl alcohol although when the fermentation is carried out at relatively alkaline pH levels, a dismutation of the acetaldehyde occurs to form ethyl alcohol and acetic acid in equimolar concentration (Neuberg's type III fermentation). Lipmann (1939) has shown that phosphate must participate in the dehydrogenation of pyruvic acid by lactic acid bacteria and the energy derived can be used to synthesize adenylic acid pyrophosphate from free phosphate and adenylic acid; Mg^{++}, Mn^{++} or Co^{++} is required. With bacteria the reactions involving pyruvic acid are more complex. Lactic acid (*d*-, *l*-, or *dl*-) may be formed by certain groups, i.e., *Lactobacillus, Streptococcus, Bacillus*; other groups

possess an essentially alcoholic mechanism, e.g., *Sarcina ventriculi* (Smit, 1930). A mixed lactic-alcoholic type of dissimilation is shown by *Thermobacterium mobile* which converts glucose into lactic acid (about 7 per cent), ethyl alcohol and CO_2 (45 per cent each) (Hoppenbrouwers, 1931). Other bacteria possess more complex mechanisms for the dissimilation of pyruvic acid.

Pyruvic acid may undergo the following changes anaerobically:

(1) $CH_3COCOOH \rightarrow CH_3CHO + CO_2$ (decarboxylation)

(2) $CH_3COCOOH + HOH \rightarrow CH_3COOH + HCOOH$ (hydrolysis)

(3) (1) followed by: $2\ CH_3CHO \begin{cases} \nearrow CH_3COOH \\ \searrow C_2H_5OH \end{cases}$ (dismutation)

(4) (1) followed by: $2\ CH_3CHO \rightarrow CH_3COCHOHCH_3$ (condensation and reduction)

$CH_3COCHOHCH_3 + 2\ H \rightarrow CH_3CHOHCHOHCH_3$

(5) $CH_3COCOOH + HOH \rightarrow CH_3COOH + 2\ H + CO_2$ (dismutation)

$CH_3COCOOH + 2\ H \rightarrow CH_3CHOHCOOH$

Reaction (1) is shown by both yeast and bacteria (*Sarcina ventriculi*) (Smit, 1930). Reaction (2) occurs apparently with many bacteria especially in the family *Bacteriaceae*. Organisms possessing hydrogenylase split the formic acid into H_2 and CO_2 (*Escherichia coli*) (Stephenson and Stickland, 1932), whereas in other species formic acid accumulates (*Eberthella* type). Reaction (3) is shown by yeast growing in a relatively alkaline medium (Neuberg and Hirsch, 1919), and probably by many bacteria. Reaction (4) may account for the formation of acetylmethylcarbinol and 2,3-butylene glycol by *Aerobacter* and yeast (Neuberg and Reinfürth, 1923). Reaction (5) is a dismutation demonstrated for the heterofermentative lactic acid bacteria by Nelson and Werkman (1936), confirmed by Krebs (1937a) for staphylococci and suggested by Quastel and Stephenson (1925) to account for the anaerobic dissimilation of pyruvic acid by *Escherichia coli*. The propionic acid bacteria bring about a dismutation of pyruvic acid to propionic acid (through lactic acid) and acetic acid and CO_2 (van Niel, 1928; Wood and Werkman, 1934).

Peters (1936) greatly increased our knowledge of the oxidation of pyruvic acid by his studies on the vitamin B_1-deficient pigeon

brain. The addition of thiamin stimulated the dissimilation of pyruvic acid. Lohmann and Schuster (1937) showed that the coenzyme of pyruvic acid decarboxylation was thiamin pyrophosphate; Lipmann (1937) then showed that thiamin pyrophosphate was involved in the dehydrogenation of pyruvic acid by an acetone preparation of *Bacterium acidificans-longissimum*. Hills (1938) reported a marked stimulation in the pyruvate metabolism of *Staphylococcus aureus* grown in thiamin-deficient media by simple addition of crystalline thiamin. Silverman and Werkman (1938, 1939a, 1939b) have shown that cell suspensions of *Propionibacterium pentosaceum* and *P. petersonii* require thiamin in the dissimilation of pyruvic acid and that there is an adaptation to the synthesis of thiamin by *P. pentosaceum* after growth in a thiamin-deficient medium. Barron and Lyman (1939) found gonococci, *Streptococcus hemolyticus* and *Staphylococcus aureus* to be stimulated in the breakdown of pyruvic acid by the addition of thiamin.

Although the dissimilation of pyruvic acid has been rather extensively studied, there is every reason to believe that there exist additional ways by which it functions in cellular metabolism of glucose. Wood and Werkman (1938) have suggested in connection with the utilization of carbon dioxide by heterotrophic bacteria that the formation of succinic acid involves pyruvic acid. They showed a direct correlation between the succinic acid formed and carbon dioxide fixed; furthermore, that sodium fluoride inhibits CO_2-fixation and that the formation of succinic acid is reduced to the same extent. This would suggest the possibility that the utilization of carbon dioxide involves a direct union with pyruvic acid. Perhaps this occurs even in photosynthesis. Should this suggestion be confirmed, it would offer an avenue for direct attack on problems of assimilation.

Aerobic dissimilation (respiration). Cellular respiration consists essentially of the dehydrogenation of the substrate molecule with transfer of hydrogen to gaseous oxygen through a series of oxidation-reduction systems involving primarily the transfer of electrons. The energy is thus liberated in a smooth continuous manner. The dehydrogenases adsorb the substrate molecules

and activate hydrogen which ultimately reaches molecular oxygen. Respiration is frequently defined so as to require the liberation of CO_2, i.e., a gaseous exchange. Such a definition cannot be rigidly adhered to since the liberation of CO_2 is purely incidental. Most respiring cells do evolve CO_2 although this is not always true. It is desirable to define respiration (aerobic dissimilation) in the sense of a process utilizing molecular oxygen as a hydrogen acceptor. The mechanism of bacterial respiration has received relatively little attention.

In the development of our concepts of respiration, the theory of the activation of the oxygen molecule was first generally accepted following the work of Bach. However, this theory of oxygen activation subsequently was found inadequate in itself. It has been revised by the Warburg school and conciliated with the Wieland theory of hydrogen activation almost simultaneously by Fleisch (1924), Szent-Györgyi (1924), Oppenheimer (1926) and Kluyver (Kluyver and Donker, 1926; Kluyver, 1931), independently. Kluyver particularly emphasizes the far-reaching effects and basic importance of the theory of the unity in the chemistry of cellular metabolism, and points out the general application of the theory of hydrogen transfer in biological oxidation both aerobic and anaerobic.

The investigations of Warburg have been concerned mainly with the rôle of iron in cellular respiration. According to his theory, iron in the form of a hematin derivative is intimately associated with the catalysis of respiration; the primary reaction is between iron and molecular oxygen and only in this manner can oxygen be used in respiration. Wieland regarded activation of the hydrogen as of primary importance, whereas Warburg (1928) believed the essential activation was that of the oxygen. Ionic iron was not effective, only iron in a complex organic form (Atmungsferment) was responsible for respiration. It is now known that the Atmungsferment is cytochrome oxidase of Keilin or very closely related. The inhibition of respiration by cyanide is plausibly explained by assuming a union of the iron compound and the cyanide to form an inactive complex. In fact Warburg developed his charcoal model on this premise. When active

charcoal is shaken with oxalic acid, an uptake of oxygen occurs with oxidation of the oxalic acid. The reaction is poisoned by cyanide or urethanes as in the case of living cells. The important point is that Warburg found the similarity between the charcoal model and the living cell lay in the presence of iron in both systems. When charcoals were made from pure cane sugar, the product was free from iron and inactive. Addition of iron salts, however, does not activate the charcoal; in addition organic nitrogen must be added along with the inorganic iron. The investigations of Warburg have done much to explain the utilization of oxygen by the living cell. They have been extended by the brilliant investigations of Keilin.

Systems of respiration. The simplest system of respiration will require a hydrogen donator (substrate), a dehydrogenase specific for the donator, and molecular oxygen as the H_2-acceptor. It is questionable whether organisms depending on such a system exist. Franke and Lorenz (1937) consider their "glucose-oxidase" which oxidizes glucose to gluconic acid to be an oxytropic dehydrogenase. Such a system is illustrated as follows, where AH_2 is the donator and O_2 the acceptor.

$$AH_2 + \tfrac{1}{2}O_2 \xrightarrow{\text{dehydrogenase}} H_2O + A$$

This may be the case in the oxidation of amino acids, and aldehydes (Dixon, 1937). Some dehydrogenases not acting directly with O_2 do so through intervention of dyes (e.g., methylene blue) or naturally occurring pigments which require no activation to accept hydrogen and pass it on to oxygen. The dyes that have been used do not occur naturally, although many bacteria contain respiratory pigments, e.g., pyocyanine, phthiocol, chlororaphine and toxoflavin. This type of respiration may be illustrated by the following scheme, where C stands for the hydrogen-carrying dye or pigment.

$$(1)\ AH_2 + C \xrightarrow{\text{dehydrogenase}} CH_2 + A$$

$$(2)\ CH_2 + \tfrac{1}{2}\,O_2 \rightarrow C + H_2O$$

However, other systems cannot employ O_2 directly as an accep-

tor of hydrogen, although they may do so indirectly; these require specific carriers for the hydrogen. Such naturally occurring carriers themselves may require enzymic activation; e.g., coenzyme I (cozymase) requires activation by diaphorase (coenzyme-factor); flavoprotein requires no such activation. Certain of the carriers function in anaerobic dissimilation, whereas others function in connection with the transfer of hydrogen to molecular oxygen. It is the latter type of carrier in which we are now interested. Moreover, in the same cell, dehydrogenation of different donators may be brought about by different carriers or combinations of carriers. Perhaps the most widely distributed and important of the respiratory hydrogen carriers is cytochrome (Keilin, 1925).

Cytochrome-cytochrome oxidase system of Keilin. Largely disregarding the controversy between Wieland and Warburg, Keilin proceeded to conciliate and extend the views of the two investigators. Keilin's rediscovery of cytochrome and his investigations elucidating the properties and behavior of the cytochrome-oxidase system constitute the third in this series of classical investigations dealing with biological oxidation-reduction.

MacMunn in 1886, while investigating absorption spectra of tissues, described absorption bands of hematin compounds which are now known to have been those of cytochrome. Severe criticisms came, however, from Hoppe-Seyler (1890) and the initial discovery of cytochrome (called myo-hematin and histo-hematin by MacMunn) fell into disrepute and was not accepted until Keilin in 1925, rediscovered and named the respiratory haemochromogen, cytochrome.

Before discussing the rôle of cytochrome in bacterial respiration, it is desirable to make a few general remarks regarding cytochrome and its function in cellular respiration.

When a heavy suspension of aerobic or facultative bacteria under anaerobic conditions is examined spectroscopically, the four bands of cytochrome are plainly visible. Keilin regards cytochrome as consisting of three separate hemochromogens, cytochromes a, b, and c, each with two bands. Three of the bands (one from each of the three cytochromes) constitute nearly coin-

cident lines and comprise band d. Bands a, b and c are separate and belong to cytochromes a, b and c respectively. Cytochrome is a hemochromogen, i.e., it is a reduced hematin combined with a protein or other organic nitrogenous group. The important point is that cytochrome is oxidized and reduced in a living cell; it acts as a hydrogen carrier, passing the hydrogen on to molecular oxygen which has been activated by an enzyme formerly called indophenol oxidase and now known as cytochrome oxidase. Apparently, the union of hydrogen with oxygen is unusual in that no peroxide is formed. With respect to oxidation and reduction, the essential part of the cytochrome molecule is the iron which can undergo oxidation from the Fe^{++} to the Fe^{+++} state. It is not pertinent at this point whether the oxygen unites with the cytochrome, perhaps it is sufficient to say that water is formed in the reduction of the oxygen by hydrogen present. The transfer of an electron from Fe^{++} to H^{+} results in Fe^{+++} and H (represented by oxidized cytochrome and water). Succinic acid is the principal donator (cf. Szent-Györgyi, 1937) in animal tissue, although it has recently been shown that hexose-monophosphate may function. Our knowledge of bacterial respiration is still too fragmentary to speculate on the occurrence of events in bacteria although succinic acid is of widespread occurrence and there is evidence of stimulation of washed cells by the addition of the succinic-fumaric acid system. Cytochrome is of general occurrence among aerobic and facultative bacteria (Tamiya and Yamagutchi, 1933; Yaoi and Tamiya, 1928; Yamagutchi, 1937; Frei, Riedmuller and Almasy, 1934) and absent from most if not all strict anaerobes. Such facultative organisms as the propionic acid bacteria contain the cytochrome-oxidase system and it appears probable that continuous culture of such forms in the presence of oxygen leads to an increased activity of the cytochrome-oxidase system.

The function of cytochrome may be illustrated as follows:

(1) $AH_2 + \text{cytochrome c} \xrightarrow{\text{dehydrogenase}} A + \text{reduced cytochrome c}$

(2) $\text{Reduced cytochrome c} + \frac{1}{2}O_2 \xrightarrow{\text{oxidase}} \text{cytochrome c} + H_2O$

The reduced form of cytochrome c unlike other hemochromogens is not oxidized spontaneously by oxygen but requires the action of a specific enzyme, cytochrome oxidase. The absorption spectrum of reduced cytochrome may be clearly distinguished in active cultures of bacteria, even in such essentially anaerobic forms as the propionic acid bacteria.

In remaining types of respiration the hydrogen may be transferred through two or more carriers. Such systems may involve flavoprotein, i.e., "yellow enzyme" of Warburg. Warburg's (cf. Warburg and Christian, 1938) terminology has been criticised by Thunberg (1935) and Ogston and Green (1935a). For a discussion and comparative table of nomenclature see Dixon (1939).

Flavoprotein. Flavoprotein comprises a protein carrier with a prosthetic group discovered by Banga and Szent-Györgyi (1932) who called it cytoflav (cf. Laki, 1933) and suggested that it played a rôle in respiration. Our knowledge of the action of flavoprotein is due largely to the work of Warburg and Christian (1933) who isolated the conjugated protein from yeast and showed that the prosthetic group is lactoflavin-5-phosphoric acid (also called riboflavin phosphate or alloxazine nucleotide) and that it does not function efficiently as a carrier until combined with a protein. The active nucleus of the prosthetic flavinphosphate group is iso-alloxazine. Flavin is iso-alloxazine combined with ribose and is commonly called riboflavin.

Kuhn, Rudy and Weygand (1936) synthesized the prosthetic group; and by combining it with Theorell's protein (1935b) Kuhn and Rudy (1936a, 1936b) showed that the complex was active.

Reduced flavoprotein is spontaneously oxidized by molecular oxygen when no other electroactive system of higher potential is present (cf. Warburg and Christian, 1933). Normally it transmits its hydrogen to oxygen by way of cytochrome c when present, since the latter step is much more rapid at the lowered oxygen tensions presumably present in the cells (Theorell, 1936). The flavoprotein functions with specific dehydrogenases which require hydrogen carriers between the substrate and the flavoprotein.

According to Ogston and Green (1935a), flavoprotein reacts with greatest activity, as a hydrogen carrier, with hexose diphosphate, hexosemonophosphate, glucose and malate as donators. The normal behavior of flavoprotein may be represented:

$$AH_2 + \text{coenzyme} + \text{flavoprotein} \xrightarrow{\text{dehydrogenases}} H_2\text{-flavoprotein} + A$$

$$H_2\text{-flavoprotein} + \text{cytochrome c} \rightarrow \text{flavoprotein} + H_2\text{-cytochrome c}$$

$$H_2\text{-cytochrome c} + \tfrac{1}{2}\,O_2 \xrightarrow{\text{oxidase}} H_2O + \text{cytochrome c}$$

In the presence of cyanide inhibition of the cytochrome oxidase, flavoprotein may perhaps, transmit hydrogen directly to oxygen although Theorell considers such a transfer as unphysiological. Since *L. delbrueckii* contains no cytochrome which can serve to accept hydrogen from the flavoprotein, its reoxidation probably depends on anaerobic hydrogen acceptors in the case of these bacteria. Flavoprotein appears to act as a hydrogen carrier between slowly reacting systems and its rôle in anaerobic dissimilation is probably that of a carrier. Coenzymes I and II reduce it. Since riboflavin phosphate can be reversibly oxidized and reduced, it behaves as an indicator.

Although previously only one flavoprotein was known, recently new ones have been discovered in which the prosthetic group is flavin-adenine-dinucleotide (coenzyme of amino acid oxidase). Haas (1938) has isolated a second flavoprotein from yeast in which the prosthetic group, like that in milk flavoprotein, is flavin-adenine-dinucleotide and the protein carrier differs from that of the original yeast flavoprotein and with which lactoflavin-phosphate is inactive. Haas obtained both fractions separately and was able to combine them to form the active flavoprotein.

Diaphorase, the dehydrogenase of coenzyme I, recently isolated by Straub (1939) and Straub, Corran and Green (1939) is a flavoprotein. The presence in animal tissue of an enzyme catalyzing the oxidation of reduced coenzymes I and II was demonstrated by Adler, Das and Euler (1937) and Dewan and Green (1938). The latter have demonstrated the presence of diaphorase in *E. coli*, *Bacillus subtilis* and *Bacterium proteus*.

Flavoprotein has been spectroscopically estimated in a number of genera, e.g., *Lactobacillus, Acetobacter, Clostridium, Flavobacterium, Escherichia* (Schütz and Theorell, 1938). Its occurrence in cells is general. Schütz and Theorell (1938) observed that *Lactobacillus delbrueckii, E. coli, Streptococcus lactis* and *Flavobacterium lacticum* did not alter their flavoprotein content when subjected to aerobic and anaerobic conditions, a finding which supports the view of Theorell (1936) that physiologically flavoprotein does not react directly with oxygen.

Wood, Andersen and Werkman (1938) have shown riboflavin to stimulate growth of *Lactobacillus* and *Propionibacterium* when added to a medium deficient in this constituent. Doudoroff (1938–39) has shown a similar stimulation of luminescent bacteria.

The investigations of Szent-Györgyi (1937) and of Krebs (1937b) have led to recognition of still another type of respiration based on fumaric acid catalysis. In the work of Szent-Györgyi, it was found that succinic acid was rapidly dehydrogenated to fumaric acid through cytochrome. These investigators believe that fumaric acid is a H_2-carrier along with oxalacetic acid. According to Krebs, fumaric acid is a carrier in the respiration of certain bacteria such as *Escherichia coli* and *Staphylococcus aureus.*

If fumaric acid is a carrier, it should, if present in sufficient quantity, carry on the oxidation of the substrate in the absence of oxygen and be recovered as succinic acid; and the rate of reaction should not be less than that in the presence of free oxygen. Furthermore, it must be shown that succinic acid occurs under physiological conditions in the dissimilation of the substrate and is oxidizable in the presence of oxygen.

The rate of the reaction can usually be determined by measuring CO_2 evolved or substrate disappearing. The reactions may be set up as follows:

(1) Glucose + fumarate $\rightarrow$ CO_2 + succinate
(2) Succinate + $\frac{1}{2}O_2$ $\rightarrow$ fumarate + H_2O

Reaction 1 must occur as rapidly as number 2.

Table 1 taken from Krebs (1937b) illustrates the point.

The final step is to show that the succinate formed will transport hydrogen to O_2 as rapidly as glucose since no reaction can

limit the rate to less than that of glucose. In fact, the rate of transport of hydrogen to oxygen from succinate is greater than the rate of glucose breakdown. Since this is true, some other reaction must limit the breakdown of glucose.

An objection which has been raised against the theory of Szent-Györgyi is based on the well known fact that the 4-carbon dicarboxylic acids are intermediary products in carbohydrate metabolism and are burned in the cell, the inference being that irre-

TABLE 1

Rates of oxidation of glucose by Bacterium coli by molecular O_2 and by fumarate (Krebs, 1937b)

40°; pH 6.8; 2 mg. (dry weight) bacteria per flask.

TIME AFTER THE ADDITION OF 0.507 MGM. GLUCOSE	CO_2 PRODUCED (μl.)	
	In the presence of O_2	Anaerobically in the presence of M/20 fumarate
minutes		
10	60.5	78
20	139	162
40	219	198
60	237	223
80	246	245
100	254	262
120	263	280
Succinic acid* found in the solution (μl.)		510

* One millimol = 22,400 μl.

versibly oxidized substances cannot function as catalysts. However, we have found a catalytic effect in the metabolism of *Micrococcus lysodeikticus* at low concentrations of fumaric acid. The increase in oxygen taken up exceeded by several times the quantity required to oxidize the fumaric acid added.

The succinate is reoxidized to fumarate by the cytochrome-oxidase system. The type may be represented:

$$AH_2 \xrightarrow{\text{dehydrogenase}} \text{coenzyme I} \xrightarrow{\text{diaphorase}}$$

$$\text{fumarate} \xrightarrow[\text{dehydrogenase}]{\text{succinic}} \text{cytochrome} \xrightarrow{\text{oxidase}} O_2$$

$$\text{flavoprotein} \longrightarrow O_2$$

Although cell respiration is the sum total of oxygen used by all types present in a cell, there is practically no knowledge of the proportional respiration accounted for by the different mechanisms.

PRINCIPAL AND SECONDARY RESPIRATION

From the standpoint of the cell, respiration is frequently classified either as (1) principal and secondary or (2) cyanide-sensitive and cyanide-insensitive. Principal respiration (Hauptatmung) is that constituting the main types in a normal cell, whereas secondary respiration (Nebenatmung) is that which constitutes relatively incidental (non-ferment hemin) respiration. In the main, principal respiration is that due to ferment-hemin (cytochrome) and is cyanide-sensitive although part of the secondary is also sensitive. The principal respiration probably includes certain reactions such as that due to flavoprotein which normally brings about oxidation through the cytochrome-oxidase system but which may transmit hydrogen directly to oxygen when the cytochrome is poisoned by cyanide. Alt (1930) has shown that cyanide-insensitive respiration is less in normal cells than in those that have been injured during the experiment. It is frequently claimed that this observation proves the abnormal character of such secondary respiration. The finding of Gourévitch (1937) that the amount of extractable flavin is proportional to cyanide-insensitive respiration indicates that this secondary respiration is due to a change in the behavior of the "yellow respiratory enzyme" to react directly with oxygen after inhibition by cyanide. It is probable that a substantial part of the secondary respiration of bacteria consists of pigment respiration. Present in bacteria are many pigments which function as carriers or acceptors, accepting hydrogen and passing it on perhaps directly or indirectly to molecular oxygen. Among the better known are pyocyanine, phthiocol and toxoflavin not to mention those such as the flavins which have been more thoroughly discussed already. Pyocyanine according to Friedheim (1931) strongly stimulates the respiration of pigment-free strains of *Pseudomonas aeruginosa* as well as of red blood cells. He found a more marked effect on

cells containing hemin systems. Such catalysis is cyanide-sensitive. Reduced pyocyanine is autoxidizable and in those cases where the oxidation is brought about by direct reaction with molecular oxygen (without iron) cyanide will exert no effect; e.g., pyocyanine respiration of the anaerobic tetanus bacteria (Frei, 1934). When pyocyanine acts as the H_2-acceptor in the respiration of bottom yeast with hexosemonophosphate as the substrate, cyanide (M/600) causes an inhibition of 31 per cent of the pyocyanine catalysis (Ogston and Green, 1935a, 1935b). The respiration of *Staphylococcus aureus* is strongly stimulated by pyocyanine (Ehrismann, 1934).

Phthiocol is a yellow pigment found in the tubercle bacillus. It exists in the bacteria in oxidized form and is a relatively negative redox system. Its effect on respiration has not been reported. *Bacterium violaceus* contains a pigment, violacein, which increases respiration of bacteria freed of their pigment. Since the reduced form is not autoxidizable, it must function as a hydrogen carrier according to Friedheim (1932). Chlororaphin, the green pigment of *Bact. chlororaphis* forms a reversible system sharing many characteristics with pyocyanine (Elema, 1933). Toxoflavin found in *Bact. bongkrek* (van Veen and Mertens, 1934) is the prosthetic group of a toxic yellow pigment and is a reversible system, electroactive between pH 4 to 8 (Stern, 1935). Phoenicein found in *Penicillium phoeniceum*, is a reversible redox system. The reduced form is autoxidizable. The fungus contains no cytochrome and its respiration may be iron-free. Phoenicein increases the respiration of *Ps. aeruginosa* several times (Friedheim, 1933). In addition, secondary oxidations by H_2O_2 with peroxidases and simple iron systems involving direct attack on the substrate may occur. These would be part of the secondary respiration but cyanide-sensitive, since peroxidases have been shown to contain iron. Finally, as part of secondary respiration there may occur a direct oxidation of unsaturated linkages especially in the fatty acids by hemin (Kuhn and Brann, 1927) or metal salts (Rosenthal and Voegtlin, 1933).

Haas (1934) has given us evidence in the case of yeast, regarding the relative intensities of the principal and secondary respirations.

Using a spectroscopic method, Haas demonstrated that in the intact cells practically all of the oxygen consumption is due to the activity of the cytochrome system.

Roman (1938) lists under "Nebenatmung"

A. Dehydrogenations
   (1) Oxytropic dehydrogenation (Schardinger reaction).
   (2) Flavoprotein
   (3) Oxyhydrogenases and oxidases: oxidative deamination of amino acids, protamines, tyrosine and uric acid.
   (4) Quinone catalyses by autoxidizable chromogens.
   (5) Secondary oxidations by H_2O_2.
      (a) Direct on the substrate.
      (b) By peroxidases and thermostable iron systems (pseudo-peroxidases).

B. Non-dehydrogenation (peroxide) oxidation as with unsaturated fatty acids; perhaps further little-known mechanisms, i.e., ω-oxidation and ring splitting (proline).

Studies on cellular metabolism have confirmed the view, originally proposed by Wieland and conciliated with the view of Warburg, especially by Kluyver, that the essential principle of cellular metabolism involves the transference of hydrogen from donator to acceptor. A series of graduated redox systems provides the mechanism of transfer and results in a smooth and regulated flow of energy for the use of the cell in assimilation. Under anaerobiosis, the ultimate transfer is limited to reducible intermediate products of dissimilation; under aerobiosis, oxygen is the final acceptor. Here the systems of Warburg and Keilin are active. Hydrogen, activated by suitable dehydrogenases and transported by carrier systems, is accepted by the inert oxygen only after activation of the oxygen molecule. This is accomplished by alternative reduction of the Fe^{+++} in the prosthetic group of hemin compounds by the hydrogen, and oxidation of the Fe^{++} by the oxygen from the air. This path must constitute, in the main, that of the principal respiration; in addition a certain uptake of oxygen occurs in the absence of the hemin compounds. Our knowledge of bacterial respiration is too inadequate to reconstruct the respiratory systems with any degree of assurance. In

the light of our present knowledge, it appears that the principles and the basic mechanisms underlying bacterial dissimilation are substantially those found in the higher, differentiated forms of life but providing for greater adaptation and variation.

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